PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 15/28, C12N 5/20
C12P 21/08, C12Q 1/24
G01N 33/53, 3/554

A1

(11) International Publication Number: WO 92/22584

(43) International Publication Date: 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/05187

(22) International Filing Date: 16 June 1992 (16.06.92)

(30) Priority data:
716,917 18 June 1991 (18.06.91) US

(71)(72) Applicants and Inventors: CAPLAN, Arnold, I. [US/US]; 1300 Oakridge Drive, Cleveland Heights, OH 44121 (US). HAYNESWORTH, Stephen, E. [US/US]; 3643 Antisdale Road, Cleveland Heights, OH 44118 (US).

(74) Agent: MINNICH, Richard, J.; Fay, Sharpe, Beall, Fagan, Minnich & McKee, 1100 Superior Avenue, Suite 700, Cleveland, OH 44114-2518 (US).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC (European patent), MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).

Published
With international search report.

(54) Title: MONOCLONAL ANTIBODIES SPECIFIC FOR MARROW-DERIVED MESENCHYMAL CELLS

(57) Abstract

The invention relates to a process for producing monoclonal antibodies which are specific to marrow-derived mesenchymal cells (i.e. mesenchymal stem cells), as well as the monoclonal antibodies that are produced by the process of the invention. In addition, the present invention is directed to the hybrid cell lines or hybridomas that synthesize and secrete these monospecific antibodies, and the use of the monoclonal antibodies for diagnostic and/or therapeutic purposes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		
ES	Spain	MG	Madagascar		

MONOCLONAL ANTIBODIES SPECIFIC FOR MARROW-DERIVED MESENCHYMAL CELLS

Background of the Invention

The present invention is directed to a process for producing monoclonal antibodies that are specific for marrow-derived mesenchymal cells (i.e. mesenchymal stem cells), as well as the monoclonal antibodies that are produced by the process of the invention. In addition, the present invention is directed to the hybrid cell lines or hybridomas that synthesize and secrete these monospecific antibodies, and the use of the monoclonal antibodies for diagnostic and/or therapeutic purposes.

Marrow-derived mesenchymal cells are the formative pluripotential blast cells found in the bone that are believed to be capable of differentiating into any of the specific types of connective tissues (i.e. the tissues of the body that support the specialized elements; particularly adipose, areolar, osseous, cartilaginous, elastic, and fibrous connective tissues) depending upon various environmental influences. Although these cells are normally present at very low frequencies in bone marrow, the inventors of the present inventions have discovered a process for isolating, purifying, and greatly replicating these cells in culture, i.e. <u>in vitro</u>. This discovery is the subject matter of a co-pending U.S. patent application.

The present invention relates to the generation of monoclonal antibodies specific to the cell surface determinants of the marrow-derived mesenchymal cells. Since there are currently no known specific markers for marrow-derived mesenchymal cells, such as enzyme activity, extracellular matrix molecules, or characteristic morphology, the monoclonal antibodies of the present invention

(which recognize cell surface determinants on marrow-derived mesenchymal cells but not certain other cells such as hemopoietic cells) provide for effective monospecific probes which can be utilized for identifying, quantifying, and/or purifying marrow-derived mesenchymal cells from tissue specimens such as bone marrow.

While the production of monoclonal antibodies by the fusion of spleen cells from immunized mice and myeloma cells grown in continuous culture has been previously described, e.g. Kohler, et al. in Nature, Vol. 256, pp. 495-497 (1975), Kohler, et al. in European Journal of Immunology, Vol. 6, pp. 511-519 (1976), Galfre, et al. in Nature, Vol. 266, pp. 550-552 (1977), and in the text Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analysis, R. Kennett, T. McKearn, K. Bechtol, Eds., Plenum Press, NY, and London, (1980), and the techniques for the chemical selection of hybridomas arising from such a fusion, and the subsequent isolation of single antibody secreting cell clones for the production of the monoclonal antibodies are also known, no cell lines have ever been produced capable of synthesizing and secreting monoclonal antibodies which not only selectively bind marrow-derived mesenchymal cells and not hemopoietic and other closely related cells, but also selectively differentiate between marrow-derived and periosteum-derived mesenchymal stem cells. The ability of these antibodies to selectively bind to specific types of mesenchymal stem cells makes them excellent probes for identifying, quantitating, isolating, and/or purifying the mesenchymal stem cells in samples of marrow, etc.

Summary of the Invention

The present invention relates to hybridomas which synthesize and secrete monoclonal antibodies that are specific for marrow-derived mesenchymal cells (i.e. mesenchymal stem cells). The antibodies recognize an antigen on the cell surface of marrow-

derived mesenchymal cells but do not recognize an antigen on hemopoietic cells. Moreover, the present invention is also directed to the monoclonal antibodies produced by the hybridomas of the invention, as well as the particular method utilized for producing both the hybridomas and the specific monoclonal antibodies.

In an additional aspect, the present invention is directed to various methods of utilizing the monoclonal antibodies produced by the present invention for therapeutic and/or diagnostic purposes.

Description of the Drawings

The following is a brief description of the drawings which are presented for the purposes of illustrating the invention and not for the purposes of limiting the same.

FIGURE 1 is a phase contrast photomicrograph of a monolayer culture of fibroblast-like cells derived from human marrow (100x);

FIGURE 2 is a series of photomicrographs (Mallory Heidehain Staining) of a histological section of a composite containing cultured human marrow fibroblasts and ceramic after two weeks of incubation in a nude mouse; FIGURE 2A shows the formation of new bone (B) lining the pores of the ceramic ghost (G) (40x); FIGURE 2B indicates that the fibrous tissue (F) was present in most of the pores whereas host vascular (V) was present in only some pores but not others (100x of boxed area in FIGURE 2A); FIGURE 2C indicates that the osteocytes (C) were clearly visibly embedded within the bone matrix. Putative osteoblasts (OB) lined the inner surface of the new bone matrix (400x of boxed area in Figure 2B);

FIGURE 3 is a series of photomicrographs (Mallory Heidehian staining) of a histological section of a composite containing cultured human marrow fibroblasts in ceramic after three weeks of incubation in a nude mouse; FIGURE 3A indicates that bone (B) was observed lining a greater number of pores of the ceramic ghost (G) than in Figure 2 (2 week incubation) (40x); FIGURE 3B demonstrates

that the fibrous tissue (F) still remained in the inner spaces of most of the pores. In addition, host vascular (V) was also present in some pores (100x of boxed area in Figure 3A);

FIGURE 4 is two photomicrographs (Mallory Heidenhain staining) of a histological section of cultured human marrow fibroblasts in ceramic after six weeks of incubation in a nude mouse; FIGURE 4A indicates that bone (B) was observed lining most of the pores of the ceramic ghost (G) (40x); FIGURE 4B shows the fibrous tissue (F) observed in the inner spaces of a few pores, however, marrow (M) and vascular (V) had replaced the fibrous tissue in a majority of the pores (100x of the boxed area in Figure 4A).

FIGURE 5 is a photograph indicating the positive results of the IgG ELISA assay of Example 2 (III)(A).

FIGURES 6A-6H are photomicrographs of a typical frozen section of pelleted culturally expanded human marrow-derived cells by indirect immunofluorescence (400 x). FIGURES 6A (phase) and 6B (fluorescence), 6C (phase) and 6D (fluorescence), and 6E (phase) and 6F (fluorescence), and 6G (phase), and 6H (fluorescence) represent SB-1 control, SH2, SH3, and SH4 antibodies which tested positive to specificity for IgG secretion and the specificity to frozen pelleted culture marrow cells.

FIGURES 7A-7H are photomicrographs demonstrating the typical positive results from the indirect immunofluorescence analysis of living cultured marrow-derived mesenchymal cells in micromass (400x). FIGURES 7A (phase) and 7B (fluorescence), 7C (phase) and 7D (fluorescence), and 7E (phase) and 7F (fluorescence), and 6G (phase) and 6H (fluorescence) represent SB-1 control, SH2, SH3, and SH4 antibodies which tested positive to specificity to living cultured marrow-derived mesenchymal cells.

Detailed Description of the Invention

The present invention relates to the discovery that through a fairly detailed process, the progenitor cells to the various types

of connective tissues can be isolated and purified from tissue such as bone marrow. These cells are referred to by the present inventor as "marrow-derived mesenchymal cells" and/or "mesenchymal stem cells". In this regard, it has been found that although these progenitor mesenchymal cells are normally present in bone marrow in very minute amounts and that these amounts greatly decrease with age (i.e. from about 1/10,000 cells in a relatively young patient to about 1/1,000,000 in an elderly patient), the progenitor or mesenchymal stem cells can be isolated from tissue and purified when cultured in a specific medium by their selective attachment to substrates. This discovery, as indicated above, is the subject of a co-pending U.S. patent application.

In addition, it has also been found that the isolated and purified marrow-derived mesenchymal cells can be grown in an undifferentiated state through mitotic expansion in a specific medium. These cells can then be harvested and activated to differentiate into bone, cartilage, and various other types of connective tissue by a number of factors, including mechanical, cellular, and biochemical stimuli.

As a result, it has been determined that the marrow-derived mesenchymal cells possess the potential to differentiate into cells which produce a wide variety of connective tissue, such as osteoblasts and chondrocytes, and possibly tendon, ligament and dermis, and that this potential is retained after isolation and for several population expansions in culture. Thus, by being able to isolate, purify, greatly multiply, and then activate the marrow-derived mesenchymal cells (or mesenchymal stem cells) to differentiate into the specific types of connective tissue desired, such as bone-forming osteoblast cells, etc., a highly effective process exists for treating skeletal and other connective tissue disorders. These findings are the subject matter of an additional U.S. patent application also co-pending with the present application.

The present invention is directed to the in vitro production of monoclonal antibodies specific to the isolated, purified, and culturally expanded marrow-derived mesenchymal cells described above, and the use of the monoclonal antibodies and associated hybridomas (i.e. the fused myeloma-lympocyte cells which produce the monoclonal antibodies and associated hybridomas) for diagnostic and/or therapeutic purposes. The monoclonal antibodies can be utilized not only for identifying, enumerating, localizing, and isolating mesenchymal stem cells through various means such as immunofluorescence, but also for delivering various pharmaceutical products, etc.

A. The Isolation and Purification of Marrow-Derived Mesenchymal Cells

Bone marrow is the soft tissue occupying the medullary cavities of long bones, some haversian canals, and spaces between trabecular of cancellous or spongy bone. Bone marrow is of two types - red, which is found in all bones in early life and in restricted locations in adulthood (i.e. in the spongy bone) and is concerned with the production of blood cells (i.e. hemopoiesis) and hemoglobin (thus, the red color); and yellow, which consists largely of fat cells (thus, the yellow color) and connective tissue.

As a whole, bone marrow is a complex tissue comprised of red and white blood cells, their precursors, and a group of cells consisting of fibroblasts, reticulocytes, adipocytes, and endothelial cells which formulate a connective tissue network called "stroma". Cells from the stroma morphologically regulate the differentiation of hemopoietic cells through direct interaction via cell surface proteins and the secretion of growth factors and are involved in the foundation and support of the bone structure. Studies using animal models have suggested that bone marrow contains "pre-stromal" cells which have the capacity to

differentiate into cartilage, bone, and other connective tissue cells. (Beresford, J.N.: Osteogenic Stem Cells and the Stromal System of Bone and Marrow, Clin. Orthop., 240:270, 1989). Recent evidence indicates that these cells, called pluripotent stromal stem cells or mesenchymal stem cells, have the ability to generate into several different types of cell lines (i.e. osteocytes, chondocytes, adipocytes, etc.) upon activation. However, the mesenchymal stem cells are present in the tissue not only in very minute amounts with a wide variety of other cells (i.e. erythrocytes, platelets, neutrophils, lymphocytes, monocytes, cosimophils, basophils, adipose cells, etc.) in an inverse relationship with age, they are capable of differentiating into an assortment of connective tissues depending upon the influence of a number of bioactive factors.

As a result, the inventors have developed a process for isolating and purifying the marrow-derived mesenchymal cells from tissue prior to differentiation and then culturally expanding the marrow-derived mesenchymal cells to produce a valuable tool for skeletal therapy. The objective of such manipulation is to greatly increase the number of potentially reparative cells and to utilize these cells to redirect and/or reinforce the body's normal reparative capacity. In this regard, the marrow-derived mesenchymal cells can then be subsequently harvested in great numbers and applied to areas of connective tissue damage to enhance or stimulate in-vivo growth for regeneration and/or repair, to improve implant adhesion to various prosthetic devices through subsequent activation and differentiation, enhance hemopoietic cell production, etc.

Along these lines, various procedures are contemplated by the inventors for transferring, immobilizing, and activating the culturally expanded, purified marrow-derived mesenchymal cells at the site for repair, implantation, etc., including injecting the cells at the site of a skeletal defect, incubating the cells with a prosthesis and implanting the prosthesis, etc. Thus, by

isolating, purifying and greatly expanding the number of cells prior to differentiation and then actively controlling the differentiation process, the culturally expanded undifferentiated marrow-derived mesenchymal cells can be utilized for various therapeutic purposes such as to elucidate cellular, molecular, and genetic disorders in a wide number of metabolic bone diseases and skeletal dysplasias.

As more specifically indicated below in Example 1, the marrow-derived mesenchymal cells isolated and purified in the present invention were derived from bone marrow attained from a number of different sources, including plugs of femoral head cancellous bone pieces obtained from patients with degenerative joint disease during hip or knee replacement surgery, and from aspirated marrow obtained from normal donors and oncology patients who have marrow harvested for future bone marrow transplantation. Although the harvested marrow was prepared for cell culture separation by a number of different mechanical isolation processes depending upon the source of the harvested marrow (i.e. the presence of bone chips, peripheral blood etc.), the critical step involved in the isolation processes was the use of a specially prepared medium which contained agents which allowed for not only marrow-derived mesenchymal cell growth without differentiation but also for the direct adherence of only the marrow-derived mesenchymal cells to the plastic or glass surface area of the culture dish. By producing a medium which allowed for the selective attachment of the desired marrow-derived mesenchymal cells which were present in the marrow samples in very minute amounts, it was possible to separate the mesenchymal stem cells from the other cells (i.e. red and white blood cells, etc.) present in the bone marrow.

In this regard, it was found that a medium consisting of BGJ_b medium (Gibco, Grand Island, NY) with tested and selected lots of 10% fetal bovine serum (J.R. Scientific, Woodland, CA, or other suppliers) was well suited for use in the present invention. This medium, which was called "complete medium", contained factors which

stimulated marrow-derived mesenchymal cell growth without differentiation and allowed for the selective attachment through specific protein binding sites, etc. of only the marrow-derived mesenchymal cells to the plastic surfaces of Petri dishes. Although the specific operating mechanism of the complete medium for producing the differential attachment is currently not well understood, research is continuing in this area.

The principal components of the BGJ_b Medium (Fitton-Jackson Modification) utilized to formulate the complete medium are set forth below:

BGJ_b Medium
(Fitton-Jackson Modification)

Component	320-2591 1X Liquid (mg/L)
Inorganic Salts:	
$NaH_2PO_4 \cdot H_2O$	90.00
$MgSO_4 \cdot 7H_2O$	200.00
KCl	400.00
KH_2PO_4	160.00
$NaHCO_3$	3500.00
NaCl	5300.00
Other Components:	
Calcium Lactate	550.00
D-Glucose	10000.00
Phenol red	20.00
Sodium acetate	50.00
Amino Acids:	
L-Alanine	250.00
L-Arginine	175.00
L-Arginine HCl	---
L-Aspartic acid	150.00
L-Cysteine $HCl \cdot H_2O$	101.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Histidine $HCl \cdot H_2O$	---
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine	240.00
L-Lysine HCl	---
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophan	40.00
L-Tyrosine	40.00
DL-Valine	65.00
L-Valine	---

Vitamins:

α-tocopherol phosphate (disodium salt)	1.00
Ascorbic acid	50.00
Biotin	0.20
D-Ca pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
i-Inositol	0.20
Nicotinamide	20.00
Para-aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04

In addition, it was also found that the medium F-12 Nutrient Mixture (Ham) (Gibco, Grand Island, NY) exhibited the desired properties for selective marrow-derived mesenchymal cell separation. The principal components of the F-12 Nutrient Mixture (Ham) are as follows:

F-12 Nutrient Mixture (Ham)

Component	320-1765 1X Liquid (mg/L)	430-1700 Powder (mg/L)
Inorganic Salts:		
$CaCl_2$ (anhyd.)	---	33.22
$CaCl_2 \cdot 2H_2O$	44.00	---
$CuSO_4 \cdot 5H_2O$	0.00249	0.00249
$FeSO_4 \cdot 7H_2O$	0.834	0.834
KCl	223.60	223.60
KH_2PO_4	---	---
$MgCl_2$ (anhyd.)	---	57.22
$MgCl_2 \cdot 6H_2O$	122.00	---
$MgSO_4$ (anhyd.)	---	---
$MgSo_4 \cdot 7H_2O$	---	---
NaCl	7599.00	7599.00
$NaHCO_3$	1176.00	---
Na_2HPO_4 (anhyd.)	---	142.04
$Na_2HPO_4 \cdot 7H_2O$	268.00	---
$ZnSO_4 \cdot 7H_2O$	0.863	0.863

Other Components:

D-Glucose	1802.00		1802.00
Hypoxanthine	4.10		---
Hypoxanthine (sodium salt)	---		4.77
Linoleic acid	0.084		0.084
Lipoic acid	0.21		0.21
Phenol red	1.20		1.20
Putrescine 2HCl	0.161		0.161
Sodium pyruvate	110.00		110.00
Thymidine	0.73		0.73

Amino Acids:

L-Alanine	8.90		8.90
L-Arginine HCl	211.00		211.00
L-Asparagine · H_2O	15.01		15.01
L-Aspartic acid	13.30		13.30
L-Cysteine	---		---
L-Cysteine HCl · H_2O		35.12	35.12
L-Glutamic acid	14.70		14.70
L-Glutamine	146.00		146.00
Glycine	7.50		7.50
L-Histidine HCl · H_2O	20.96		20.96
L-Isoleucine	3.94		3.94
L-Leucine	13.10		13.10
L-Lysine HCl	36.50		36.50
L-Methionine	4.48		4.48
L-Phenylalanine	4.96		4.96
L-Proline	34.50		34.50
L-Serine	10.50		10.50
L-Threonine	11.90		11.90
L-Tryptophan	2.04		2.04
L-Tyrosine	5.40		---
L-Tyrosine (disodium salt)	---		7.78
L-Valine	11.70		11.70

As indicated above, the complete medium can be utilized in a number of different isolation processes depending upon the specific type of initial harvesting processes used in order to prepare the harvested bone marrow for cell culture separation. In this regard, when plugs of cancellous bone marrow were utilized, the marrow was added to the complete medium and vortexed to form a dispersion which was then centrifuged to separate the marrow cells from bone

pieces, etc. The marrow cells (consisting predominantly of red and white blood cells, and a very minute amount of mesenchymal stem cells, etc.) were then dissociated into single cells by passing the complete medium containing the marrow cells through syringes fitted with a series of 16, 18, and 20 gauge needles. It is believed that the advantage produced through the utilization of the mechanical separation process, as opposed to any enzymatic separation process, was that the mechanical process produced little cellular change while an enzymatic process could produce cellular damage particularly to the protein binding sites needed for culture adherence and selective separation, and/or to the protein sites needed for the production of monoclonal antibodies specific for said marrow-derived mesenchymal cells. The single cell suspension (which was made up of approximately 50-100 x 10^6 nucleated cells) was then subsequently plated in 100 mm dishes for the purpose of selectively separating and/or isolating the marrow-derived mesenchymal cells from the remaining cells found in the suspension.

When aspirated marrow was utilized as the source of the marrow-derived mesenchymal cells, the marrow stem cells (which contained little or no bone chips but a great deal of blood) were added to the complete medium and fractionated with Percoll (Sigma, St. Louis, MO) gradients more particularly described below in Example 1. The Percoll gradients separated a large percentage of the red blood cells and the mononucleate hemopoietic cells from the low density platelet fraction which contained the marrow-derived mesenchymal stem cells. In this regard, the platelet fraction, which contained approximately 30-50 x 10^6 cells was made up of an undetermined amount of platelet cells, 30-50 x 10^6 nucleated cells, and only about 50-500 marrow-derived mesenchymal cells depending upon the age of the marrow donor. The low density platelet fraction was then plated in the Petri dish for selective separation based upon cell adherence.

In this regard, the marrow cells obtained from either the cancellous bone or iliac aspirate (i.e. the primary cultures) were

grown in complete medium and allowed to adhere to the surface of the Petri dishes for one to seven days according to the conditions set forth in Example 1 below. Since no increase in cell attachment was observed after the third day, three days was chosen as the standard length of time at which the non-adherent cells were removed from the cultures by replacing the original complete medium with fresh complete medium. Subsequent medium changes were performed every four days until the culture dishes became confluent which normally required 14-21 days. This represented 10^3-10^4 fold increase in undifferentiated mesenchymal stem cells.

The cells were then detached from the culture dishes utilizing a releasing agent such as trypsin with EDTA (ethylene diaminetetra-acetic acid) (0.25% trysin, 1 mM EDTA (1X), Gibco, Grand Island, NY) or a chelating agent such as EGTA (ethylene glycol-bis-(2-amino ethyl ether) N,N'-tetraacetic acid, Sigma Chemical Co., St. Louis, MO). The advantage produced through the use of a chelating agent over trypsin was that trypsin could possibly cleave off a number of the binding proteins of the mesenchymal stem cells. Since these binding proteins contain recognition sites, when monoclonal antibodies were sought to be produced, a chelating agent such as EGTA as opposed to trypsin, was utilized as the releasing agent. The releasing agent was then inactivated and the detached cultured undifferentiated stem cells were washed with complete medium for subsequent use.

In this regard, the bone and cartilage lineage potentials (i.e. osteo-chondrogenic potential) of fresh and expanded marrow-derived mesenchymal cells under the influence of various bioactive factors were determined using two different in-vivo assays in nude mice. See Example 1 below. One assay involved the subcutaneous implantation of porous calcium phosphate ceramics loaded with cultured marrow-derived mesenchymal cells; the other involved peritoneal implantation of diffusion chambers inoculated with cultured marrow-derived mesenchymal cells. Whole marrow and Percoll gradient separated aspirate fractions were also analyzed in

these in-vivo assays. Histological evaluation showed bone formation in the ceramics implanted with the cultured mesenchymal stem cells derived from the femoral head and the iliac crest. No cartilage was observed in any of the ceramic grafts. In contrast, the same cells failed to form any bone or cartilage in the diffusion chambers. While whole marrow has now been shown to form bone when placed as a composite graft with ceramics in a subcutaneous site in nude mice, the amount of bone produced is substantially less than that seen when culture-expanded marrow-derived mesenchymal cells are used.

These results indicated that under certain conditions, culturally expanded mesenchymal cells have the ability to differentiate into bone when incubated as a graft in porous calcium phosphate ceramics. Although the internal factors which influence the mesenchymal stem cells to differentiate into bone as opposed to cartilage cells are not well known, it appears that the direct accessibility of the mesenchymal cells to growth and nutrient factors supplied by the vasculature in porous calcium phosphate ceramics, as opposed to the diffusion chamber, influenced the differentiation of the mesenchymal stem cells to bone.

As a result, the isolated and culturally expanded marrow-derived mesenchymal cells can be utilized under certain specific conditions and/or under the influence of certain factors, to differentiate and produce the desired cell phenotype needed for connective tissue repair or regenerative and/or to the implantation of various prosthetic devices. For example, using porous ceramic cubes filled with culture-expanded human marrow-derived mesenchymal stem cells, bone formation inside the pores of the ceramics has been generated after subcutaneous incubations in immunocompatible hosts. In a recent study conducted by the inventor's lab, i.e. Ohgushi, H., Goldberg, V., and Caplan, A. Acta Scandia., 60:334-339, 1989, rat marrow in a composite graft with porous ceramic was used to fill a segmental defect in the femur of the rat. Bone was

shown to fill the pores of the ceramic and anchor the ceramic-marrow graft to the host bone.

B. The Production of Monoclonal Antibodies Specific for Marrow-Derived Mesenchymal Cells

The present invention is directed to the *in vitro* production of monoclonal antibodies specific to the marrow-derived mesenchymal cells (mesenchymal stem cells) which were isolated, purified and culturally expanded as indicated above, and the use of the monoclonal antibodies and associated hybridomas (i.e. the fused myeloma-lymphocyte cells which produce the monoclonal antibodies) for diagnostic and/or therapeutic purposes. The monoclonal antibodies can be utilized not only for identifying, enumerating, localizing, and isolating mesenchymal stem cells through various diagnostic means such as immunofluorescence, but also for delivering various pharmaceutical products, and additional therapeutic uses.

The marrow-derived mesenchymal cells utilized in the invention for immunization were isolated and purified according to the procedure set forth above with the exception that after the first passage of cells that were grown to confluence, the cells were released from the surface of the Petri dish with 0.5 mM EGTA (ethylene glycol-bis-(2-amino ethyl ether) N,N'-tetraceticacid, Sigma Chemical Co., St. Louis, MO) in Moscona's Salt Solution (Ca-Mg-free Tyrode's salt solution) for one hour. In this regard, because EGTA acts as a releasing agent possibly through the chelation of Mg^{+2} and Ca^{+2} ions, the marrow-derived mesenchymal cells was first soaked with Moscona's (a saline solution containing no Mg^{+2} or Ca^{+2} ions) reagent in order to remove the Mg^{+2} and Ca^{+2} present in the complete medium, and then were incubated in EGTA for one hour. The marrow-derived mesenchymal cells were then rinsed with Tyrode's Salts (Catalog No. T-2145, without sodium bicarbonate, Sigma Chemical Co., St. Louis, MO), a balanced salt

solution which provides certain physiological factors required for the maintenance of the structure integrity of cells in vitro, and reconstituted in Tyrode's salts for injection into a mouse for the production of antibodies.

Along this line, prior to immunization (or stimulation), the marrow-derived mesenchymal cells were dissociated into single cells by passage through a 20 gauge needle. A female mouse (CBGF1/J, Jackson Labs, Bar Harbor, Maine) approximately 14 weeks old at the beginning of the immunization process, was immunized with the culturally expanded, isolated marrow-derived mesenchymal cells by peritoneal injection according to the specific immunization protocol more clearly set forth below in Example 2. In this regard, since at least one previous attempt failed to produce any monoclonal antibodies specific to the marrow-derived mesenchymal cells during immunization, several factors were explored with respect to the optimal immunization procedure in order to produce anti-mesenchymal stem cell secreting hybridomas including (1) cell disassociation (i.e. the cells were dissociated with EGTA and other agents instead of trypsin), (2) the immunizing antigen concentration, (3) the source of the purified marrow-derived mesenchymal cells utilized (i.e. cells from several different marrow donors were used to maximize the proportion of common antigens over donor-specific antigens), etc. The procedure which was discovered to produce the optimal results is set forth in detail in Example 2.

Briefly, however, approximately 2.0 x 10^6 culturally expanded marrow-derived mesenchymal cells were initially intraperitoneally injected into the mouse. The initial injection was followed by four booster injections of marrow cells obtained from multiple human donors prepared in a similar fashion spaced approximately a week apart. Marrow cells from multiple donors were utilized for the purposes of producing monoclonal antibodies specific for common epitopes found on the surface of the marrow-derived mesenchymal cells. After the 25th day, blood was drawn from the mouse and the

serum from the blood was assayed by indirect immunofluorescence in order to check that the immunization regiment had been successful in generating an immune response in the mouse to the cultured marrow-derived mesenchymal cells.

After successfully completing the four week immunization process, the mouse was sacrificed and its spleen cells (particularly the antibody-secreting plasma cells, i.e. lymphocytes, which the producing antibodies with the specificity for the marrow-derived mesenchymal cells) were fused with SP 2/0 myeloma cells (immortal antibody-secreting tumor cells obtained from Dr. Douglas Fambrough at the Carnegie Institute in Baltimore, Maryland) according to the very specific fusion procedure set forth below. The SP 2/0 myeloma cells utilized were sensitive to hypoxanthine-aminopterm-thymidine (HAT) medium by virtue of their lacking enzymes such as thymidine kinase (TK). As a result, the SP 2/0 myeloma cells died when exposed to HAT selective medium. This allowed for the selection of hybrids to be accomplished by growing the fused and unfused cells in HAT medium. Moreover, in addition to the SP 2/0 myeloma cells sensitivity to HAT medium, these cells also synthesized no immunoglobulin. The benefit of using a non-immunoglobulin secreting myeloma cell for fusion was that any immunoglobulin associated with the growth of the hybridomas arising from the fusion would indicate only a contribution from the spleen cells.

The process utilized for cell fusion to produce the hybridomas is described in detail in Example 2 below. Generally, however, the spleen cells were first mixed with one-third of the number of the myeloma cells and centrifuged. The cell pellet was then exposed to polyethylene glycol (PEG 1500, Boehringer Mannheim, W. Germany) which promoted cell fusion. The PEG was then subsequently diluted out, and the fusion mixture was centrifuged and the pellet resuspended in specific growth medium (i.e. the HAT medium which killed the myeloma cells while permitting the growth of the hybridomas) with feeder cells (such as mouse peritoneal macrophages

which aided in the establishment of hybridomas), plated into aliquots, and incubated according to the feeding and changing schedule set forth in Example 2. After approximately seven to ten days, small clusters or colonies of hybridoma cells (i.e. the immortalized progeny of cell fusion between the tumor cells and the β-lymphocytes found in the mouse spleen) appeared in the wells. Since the myeloma cell line utilized (i.e. SP 2/0 myeloma cells) was a mutant that had lost its ability to produce its own antibodies, the resultant hybridomas secreted only the antibodies of the antibody-secreting plasma cells (β-lymphocytes) obtained from the immunized mouse.

In order to identify those hybridoma colonies which synthesized and secreted antibodies having the specificity for the culturally expanded, isolated, and purified marrow-derived mesenchymal cells, a number of preliminary screening techniques (i.e. Enzyme-linked Immunosorbent Assay, Indirect Immunofluorescence Assay, etc.) were utilized to characterize the antibodies which were secreted by the various hybridomas. The positive controls for all assays included sera from the immunized mice at various dilutions obtained at the time of spleen removal.

Specifically, a series of assays were performed in order to identify hybridomas which secreted antibodies with the IgG isotope. Since this isotope was the major secreting antibody and was also the easiest to purify and use, it was preferred over IgM and IgA isotopes which may have also contained the specificity for the desired epitope. As indicated below in Example 2, out of the 764 wells which exhibited hybridoma growth (out of 960 wells initially plated), 245 of these wells tested positive for secreting antibodies with the IgG isotope.

The culture supernatant from colonies which screen positive for the production of IgG antibodies were then screened against frozen sections of pelleted marrow-derived mesenchymal cells by indirect immunofluorescence. This assay was performed in order to identify antibodies which bonded only to epitopes on the marrow-

derived mesenchymal stem cells. Out of the 245 wells which screened positive for the production of IgG antibodies, only 171 of these wells tested positive for binding with the marrow-derived mesenchymal cells.

Since the above screening step was directed to frozen sections of pelleted cultured marrow-derived mesenchymal cells, it fails to differentiate those hybridomas which secreted antibodies only specific to the surface of the cell as opposed to the intracellular portion of the cell, the hybridoma supernatants which tested positive in regard to the reactivity for IgG and frozen sections of pelleted cultured marrow-derived mesenchymal cells, were then incubated with live cultured human marrow-derived mesenchymal cells in micromass cultures (i.e. the cultured cells were replated into small masses located in the center of the tissue culture dishes where the cells remained viable and replicated) and the reactivity was measured by indirect immunofluorescence. Since the cells that were being analyzed were living cells, this assay identified antibodies which bonded to the surface of the cell and gave negative results to antibodies which bonded only to intracellular epitopes. This was important because for the monoclonal antibodies to be utilized as a useful differentiation marker, they needed to be specific to the cell surface.

As indicated below, out of 171 wells which tested positive for reactivity with pelleted cultured marrow-derived mesenchymal cells, only 15 of these wells tested positive for reactivity with living cultured marrow-derived mesenchymal cells.

The hybridomas which tested positive to each of the above preliminary screens (i.e. 15 out of 960 initial wells) were then cloned by limited dilution to ensure that the subsequent screening was performed on hybridomas which originated only from a single clone. In this regard, since multiple cell lines may have been present in the original "parent" wells (i.e. in the original fusion well, the hybridoma cells may have descended from several fusion products and/or the gradual loss of chromosomes during the initial

days after fusion may also have generated additional heterogenecity) cloning of the cells was required to obtain a monoclonal hybridoma cell line. While a detailed description of the cloning process utilized is set forth below in Example 2, the cloning process basically involved the steps of replating the cells present in the original parent wells at a density of less than one cell per well. As a result of the establishment of the single cell cultures, any colony which grew in the wells was the progeny of only the original replated cells. In order to assure monoclonality, multiple clonings were performed until 100% of the subclones were positive for two generations.

The cell lines or clones were then progated in tissue cultures or in-vivo in syngeneic or immunocompatible hosts where they continued to synthesized and secret antibodies to the purified marrow-derived mesenchymal cells. These antibodies were then recovered from the tissue culture cells or the ascite fluids by conventional techniques such as precipitation, ion exchange, affinity chromatography, etc.

The cloned hybridomas were then subsequently screened against a series of mesenchymal and non-mesenchymal derived tissues in order to identify the degree of specificity of the monoclonal antibodies to the cultured marrow-derrived mesenchymal cells. Specifically, in order to determine that the monoclonal antibodies were not specific (i.e. did not react) to the hemopoietic lineage cells in marrow (and thus could be utilized to differentiate mesenchymal tissue from the hemopoietic cells), whole marrow and several partial fractionations of the marrow were screened against hybridoma culture supernatant by indirect immunofluorescence. The results are set forth below in Table 5 (see Example 2).

In addition, in order to determine if the monoclonal antibodies reacted to epitopes common to marrow-derived mesenchymal cells and differentiated mesenchymal tissues, frozen sections of a variety of mesenchymal tissues that were obtained at surgery or autopsy were screened against the hybridoma culture supernatant by

indirect immunofluorescence. The positive and negative reactivities were noted and are set forth in Table 5.

Furthermore, in order to identify hybridomas which secreted antibodies which were only specific for mesenchymal stem cells and/or their lineage descendants (i.e. antibodies which failed to react to non-mesenchymal derived tissue), the hybridoma culture supernatants were incubated with sections of non-mesenchymal derived tissue and the antibodies reactivity was analyzed by indirect immunofluorescence. The results are also set forth in Table 5 below.

An analysis of the data indicated that three of the hybridomas produced, identified, and cloned by the present invention (i.e. SH2, SH3, and SH4) were useful for the analysis of marrow-derived mesenchymal cells. All three of these hybridomas secreted antibodies which reacted to cell surface epitopes on 99-100% of cells in the assays of the culture expanded marrow-derived mesenchymal cells. In contrast, each of the three hybridomas secreted antibodies which reacted to less than 1% of the cells in assays of whole marrow. The ability of these antibodies to selectively bind marrow-derived mesenchymal cells and not hemopoietic cells make them excellent probes for quantitating the number of mesenchymal stem cells in samples of marrow and for purifying mesenchymal stem cells from marrow.

In addition, all three of the hybridomas showed mostly negative cross reactivity when screened against a variety of mesenchymal and non-mesenchymal derived tissues, although some cross reactivity was observed with each. Of particular interest, SH3 and SH4 cross reacted to cell surface determinants on cultured cells derived from human periosteum. In this regard, the inventors previously demonstrated that periosteum was another source of mesenchymal cells and the cross reactivity of the above antibodies to cell surface epitopes on periosteal cells suggested a structural relationship between the marrow derived mesenchymal cells and periosteum derived mesenchymal cells. The SH2 antibody, however,

did not react to periosteum-derived mesenchymal cells even though it did bind to marrow derived mesenchymal cells. This selectivity makes the SH2 antibody useful for distinguishing between marrow derived and periosteum derived mesenchymal stem cells. The selectivity of the SH2 antibody coupled with the cross reactivity of SH3 and SH4 suggests that marrow derived and periosteum derived mesenchymal stem cells are related but not identical.

Deposits of the cell line cultures identified as SH2, SH3, and SH4 are on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, and are assigned the ATCC accession numbers HB 10743, HB 10744, and HB 10745, respectively. The deposits are for the purpose of enabling disclosure only and are not intended to limit the concept of the present invention to the particular materials deposited.

The results indicated that the generated monoclonal antibodies recognized cell surface determinants on the marrow-derived mesenchymal cells but not certain other closely related cells such as hemopoietic cells, etc. Since there are no specific markers for marrow-derived mesenchymal cells, the generated monoclonal antibodies now provide for effective mono-specific probes which can be utilized for identifying, quantifying, and purifying marrow-derived mesenchymal cells.

More particularly, the monoclonal antibodies produced can be labelled with suitable radioactive, enzymatic, or fluorescent labels by conventional methods and/or bound to suitable solid carriers, which should be apparent to those skilled in the art. In this regard, Example 3 below demonstrates the effectiveness of the generated monoclonal antibodies for identifying and/or quantifying the presence of marrow-derived mesenchymal cells in biological samples such as bone marrow with conventional immunological methods. For example, the monoclonal antibodies can be used in combination with, or coupled to, an immunochemical such as fluorescein isothiocynate, peroxidase, alkaline phosphates, or other such markers. Moreover, the monoclonal antibodies can be

bound or attached to certain substrates and utilized to capture marrow-derived mesenchymal cells when tissue samples such as bone marrow, are brought in contact with the attached monoclonal antibodies. The bound cells may then be separated from the solid phase by known methods depending essentially upon the nature of the solid phase and the antibody. The unbound cells can be recovered and used for various therapeutic purposes such as for the regeneration of bone, etc., depending upon the various external and internal factors.

As a result, the present invention contemplates any method of employing monoclonal antibodies to separate marrow-derived mesenchymal cells from other cells such as hemopoietic cells in bone marrow. For example, a further embodiment of the present invention is directed to a method of producing a population of marrow-derived mesenchymal cells comprising the steps of providing a cell suspension of tissue containing bone marrow; contacting the cell suspension with monoclonal antibodies which recognize an epitope on the marrow-derived mesenchymal cells but do not recognize an epitope on the hemopoietic cells; and separating and recovering from the cell suspension the cells bound by the monoclonal antibodies.

In addition, an alternate embodiment of the invention is directed to a method of providing a population of marrow-derived mesenchymal cells comprising the steps of providing a cell suspension of tissue containing bone marrow; contacting the cell suspension with solid-phase linked monoclonal antibodies which recognize an epitope on the cell surface of the marrow-derived mesenchymal cells but do not recognize an epitope on the hemopoietic cells; separating the unbound cells from the solid-phase linked monoclonal antibodies; and, recovering the bound cells from the liquid linked monoclonal antibodies.

The following examples are included for the purposes of further illustrating the detailed steps of the present invention.

EXAMPLE 1

The Isolation, Purification and Cultural Expansion of Marrow-Derived Mesenchymal Cells

Marrow Harvest

Marrow in femoral head cancellous bone pieces was obtained from patients with degenerative joint disease during hip or knee joint replacement surgery. In addition, marrow was also obtained by iliac aspirate from normal donors and oncology patients who were having marrow harvested for future bone marrow transplantation. All of the oncology patients had malignancies unrelated to the stromal cells and the stromal cells expressed normal karyotype.

Preparation of Marrow for Cell Culture

A. From Plugs of Cancellous Bone Marrow

Plugs of cancellous bone marrow (0.5-1.5 ml) were transferred to sterile tubes to which 25 ml BGJ_b medium (GIBCO, Grand Island, NY) with selected batches of 10% fetal bovine serum (JR Scientific, Woodland, CA) (complete medium) was added. The tubes were vortexed to disperse the marrow then spun at 1000 x RPM for 5 minutes to pellet cells and bone pieces. The supernatant and fat layer were removed and the marrow and bone were reconstituted in 5 ml complete medium and vortexed to suspend the marrow cells. The suspended cells were collected with a 10 ml syringe fitted with an 16 gauge needle and transferred to separate tubes. Bone pieces were reconstituted in 5 ml. Complete medium and the marrow cells were collected as before. Collection of marrow cells was considered complete when a pellet of yellowish-white cancellous bone pieces was all that remained in the original tube. Marrow cells were separated into a single cell suspension by passing them through syringes filled with 18 and 20 gauge needles. Cells were spun at 1000 x RPM for 5 minutes after which the fat layer and supernatant were removed. Cells were reconstituted in complete medium, counted

with a hemocytometer (red blood cells were lyzed prior to counting with 4% acetic acid), and plated in 100 mm dishes at 50-100 x 10^6 nucleated cells/dish.

B. From Aspirate Bone Marrow

Aspirate marrow (5-10 ml) was transferred to sterile tubes to which 20 ml complete medium was added. The tubes were spun at 1000 x RPM for 5 minutes to pellet the cells. The supernatant and fat layer were removed and the cell pellets (2.5-5.0 ml) were loaded onto 70% Percoll (Sigma, St. Louis, MO) gradients and spun at 460 x g for 15 minutes. The gradients were separated into three fractions with a pipet: top 25% of the gradient (low density cells-platelet fraction), pooled density = 1.03 g/ml; middle 50% of the gradient (high density cells-mononucleated cells), pooled density = 1.10 g/ml; and, bottom 25% of the gradient (red blood cells), pooled density = 1.14 g/ml. In preliminary experiments each of these three pools were plated separately in complete medium in 100 mm dishes. Adherent cells were observed to be localized to the low density cells. To produce adherent cell cultures for all subsequent experiments only the low density cells were plated.

Culturing and Passaging of Marrow Stromal Cells

Marrow cells from either the femoral head cancellous bone or the iliac aspirate were cultured in complete medium (i.e. BGI_b medium with 10% fetal bovine serum) at 37°C in humidified atmosphere containing 95% air and 5% CO_2. In preliminary experiments the cells were allowed to attach for 1, 3, or 7 days prior to the initial medium change. No increase in cell attachment was observed after day 1, therefore one day was chosen as the standard length of time at which nonadherent cells were removed from the cultures by replacing the original medium with 7 ml of fresh complete medium. Subsequent medium changes were performed every 4 days. When culture dishes became confluent, the cells were detached with 0.25% trypsin with 0.1 mM EDTA (GIBCO) for 10-15

minutes at 37°C. The action of trypsin was stopped with 1/2 volume fetal bovine serum. The cells were counted, split 1:3, and replated in 7 ml complete medium. Aliquots of cells were cryopreserved in 90% fetal bovine serum with 10% DMSO (freezing medium).

Preparation of Cultures for In Vivo Incubations in Ceramics and Diffusion Chambers

Cultured cells were detached from plates as described for subculturing. After inactivating the trypsin, the cells were washed twice with 10 ml serumless BGJ_b medium, counted, and then adjusted to the appropriate concentration with serumless BGJ_b. Whole marrow and Percoll fractions were rinsed twice with 10 ml serumless BGJ_b and adjusted to the appropriate concentration with serumless BGJ_b. Porous ceramic cubes (3 mm^3) composed of 60% hydroxyapatite + 40% β-tricalcium phosphate (Zimmer Corporation, Warsaw, Indiana) were added to the cell suspensions under slight vacuum and soaked for up to 90 minutes prior to surgical implantation.

Diffusion chambers were constructed of lucite rings and Millipore filters as described elsewhere (Ashton, et al., 1980). Cells were prepared as described above and added to the chambers in 100-140 ul of serumless BGJ_b medium. Chambers were sealed with a drop of cement and immersed in serumless BGJ_b for up to 90 minutes prior to surgical implantation.

Surgical Implantation of Ceramics and Diffusion Chambers

Ceramics - Nude mice (National Institute of Health, nu/nu strain) were anesthetized with ether and placed on their stomachs. Four small longitudinal incisions (5 mm) were made along the backs. Ceramic-marrow grafts were inserted into the pockets and positioned as lateral in the pockets as possible. Incisions were closed with Autoclips (Becton Dickenson and Company, Parsippany, NJ). Each pair of pockets received a different pair of ceramic-marrow graft

so that four different samples (2 ceramic cubes per sample) were incubated per mouse.

Diffusion Chambers - Nude mice were anesthetized with ether and placed on their backs. Incisions were made through the skin and peritoneum, and diffusion chambers were inserted into the peritoneal cavity. The peritonea were closed with sutures and the skin, with Autoclips. Only one chamber was inserted per mouse and it contained cultured cells identical to cells loaded in one of the four pairs of the ceramic-marrow grafts implanted into the same mouse.

Histological Evaluation

Nude mice were sacrificed and the ceramic-marrow grafts harvested 1-8 weeks after implantation (Table 1 and Table 3). Ceramic were fixed in 10% buffered formalin, demineralized for 7 hours in RDO Rapid Bone Decalcifier (Dupage Kinetics Laboratories, Inc., Plainfield, Illinois), embedded in paraffin, serial sectioned (5 um thick), and stained with Mallory Heidenhain or Toluidine blue.

Diffusion chambers were harvested 3-10 weeks after implantation (Table 2 and Table 3). Chambers were fixed in 10% buffered formalin, paraffin embedded, serially sectioned, and stained with Mallory Heidenhain or Toluidine blue.

TABLE 1
Incubation of Composite Graphs of Cultured Human Marrow Cells and Ceramic in Nude Mouse

					Weeks Postimplantation							
Donor #	Age/Sex	Site	Pass #	Conc.	1	2	3	4	5	6	7	8
1	50/M	FH	4	$20x10^6$						+		
1	50/M	FH	5	$2x10^6$			–		+	+		
1	50/M	FH	6	$0.7x10^6$			+	+				+
2	65/M	FH	1	$5x10^6$					++	+++		
2	65/M	FH	2	$4x10^6$			+++			+++		+++
3	65/M	FH	P	$6x10^6$	–	+				+++	+++	+++
4	66/F	FH	1	$10x10^6$			+		+++	+++		+++
5	64/M	FH	P	$8x10^6$	–	+	++			+++		+++
6	64/M	FH	1	$4x10^6$		+				+++		+++
7	67/F	FH	P	$7x10^6$	–	+	++	+++				
8	34/F	IC	1	$4x10^6$			–			+		
9	42/M	IC	P	$4x10^6$			–			–		
10	38/M	IC	P	$4x10^6$			–			++		
11	45/M	IC	P	$4x10^6$			+			++		

Pass # is the number of subcultures
Conc. is the concentration of cells in cells/ml
Site is the site of marrow harvest
FH - femoral head
IC - Iliac crest
P - primary cultures

– = None of the pores contained bone
+ = 0-30% of the pores contained bone
++ = 30-70% of the pores contained bone
+++ = greater than 70% of the pores contained bone

TABLE 2

Incubation of Cultured Human Marrow Cells in Diffusion Chambers

Donor	Age/Sex	Site	Pass #	Cells/Chamber	3	6	8	10
1	50/M	FH	4	$4x10^6$		-		
2	65/M	FH	1	$3x10^6$		-		
3	65/F	FH	P	$4x10^6$		-	-	
4	66/F	FH	1	$4x10^6$	-			-
5	64/M	FH	P	$4.5x10^6$		-		

P = primary culture
Pass # = the number of subcultures
Site = the site of marrow harvest
FH = femoral head
IC = iliac crest
\- = no bone in chamber
\+ = 0-30% of chamber contains bone
++ = 30-70% of chamber contains bone
+++= more than 70% of chamber contains bone

RESULTS

In Vitro Cultures

Adherent marrow-derived mesenchymal cells from femoral head cancellous bone or iliac aspirate have similar morphology, almost all being fibroblastic, with few adipocytic, polygonal or round cells (Figure 1). Histochemical staining for alkaline phosphatase yields variable positive reactivity with no noticeable difference between cells derived from cancellous bone marrow or aspirate marrow. Adherent cells from both harvest sites fail to produce an extracellular matrix which stains metchromatically with Toluidine blue or positive for von Kossa; a positive staining would have indicated the possibility that cartilage or bone tissue was produced in these cultures.

In Vivo Incubation of Cultured Marrow Cells with Ceramics

Calcium phosphate ceramic blocks were soaked in culture medium containing various concentrations of cultured marrow-derived mesenchymal cells from either femoral head cancellous bone or iliac

aspirate. The marrow donors included both males and females ranging in age from 34 to 67 years old (Table 1). Cells from primary culture and first through sixth passage were assayed, with the cell-loading concentration ranging from 0.7 x 10^6 to 20 x 10^6 cells/ml. Marrow-derived mesenchymal cell-loaded ceramic blocks were surgically implanted subcutaneously into nude mice and incubated from 1 to 8 weeks. Upon harvest, ceramics were fixed, demineralized and the presence of bone and cartilage was determined by histological evaluation. Table 1 summarizes the data.

Bone, but not cartilage, was observed in the pores of each graft of ceramics and cultured marrow-derived mesenchymal cells from femoral head cancellous bone. The earliest bone was observed at 2 weeks in less than 30% of the pores of each ceramic (Figure 2). At three weeks, the number of pores containing bone varied from less than 30% to greater than 70% (Figure 3). By six weeks, the majority of the ceramics contained bone in greater than 70% of the pores (Figure 4). No obvious correlation could be made between the age of the donors and the amount of bone formation. In contrast, passage number appeared to have some influence on the amount of bone formation, with primary cultures and early passaged cells (1st-2nd passages) giving more bone formation than late passaged cells (4th-6th passages). Bone formation appears to begin with osteoblast differentiation and bone deposition onto the surfaces of the ceramic pores and appears to progress towards the center of the pores as cells lining the surface of the new bone matrix secrete osteoid on top of previously deposited matrix. Maintenance of ceramic marrow-derived mesenchymal cell grafts for periods of 6-8 weeks resulted in bone remodeling and the identification of marrow elements in the inner spaces of each pore (Figure 2C).

Grafts of ceramics and cultured marrow-derived mesenchymal cells from iliac aspirate produced bone in three of the four samples tested (Table 1). Cartilage was not observed in any of the grafts. Bone formation in the three positive grafts was less than

that observed from ceramics grafted with cultured cells from femoral head cancellous bone marrow. Less than 30% of the pores contained bone at 3 weeks and 30-70% of the pores contained bone at 6 weeks. The remainder of the pores contained fibrous tissue and vasculature of, in all likelihood, host origin.

<u>In Vivo Incubation of Cultured Marrow Cells in Diffusion Chambers</u>

The osteo-chondrogenic potential of cultured marrow-derived mesenchymal cells was also assayed by loading cells in diffusion chambers and surgically implanting them intraperitonelly into nude mice. The cells were obtained from the same cultures used in the ceramic assays (Table 2), and the diffusion chambers were implanted into the peritonea of the same nude mice which received subcutaneous ceramic-marrow-derived mesenchymal cell grafts. After incubations for 3-10 weeks, the chambers were harvested and the presence of bone and cartilage formation determined by histological evaluation. In contrast to the presence of bone in grafts of ceramic and cultured cells from cancellous bone, no bone or cartilage was observed in any of the diffusion chambers containing cultured cancellous bone marrow-derived mesenchymal cells even after 10 weeks incubation (Table 2). Cultured iliac aspirate marrow-derived mesenchymal cells also failed to produce bone or cartilage in the diffusion chambers. Instead, hypocellular sparse fibrous tissue was observed in most of the chambers.

<u>Discussion</u>

In this example, human marrow-derived mesenchymal cells were shown to reproducibly exhibit osteogenic potential following their mitotic expansion in culture when assayed in porous calcium phosphate ceramics in nude mice. Osteogenesis was not observed when the same cells were incubated in diffusion chambers in the same nude mice. Collectively, these data show that human marrow contains cells, which can be selected and expanded in culture,

which have the potential to differentiate into bone when incubated in vivo as a graft in porous calcium phosphate ceramics.

The absence of bone formation in diffusion chambers suggests that the ceramics assay may be a more sensitive assay for differentiation of bone from marrow cells. Bab, et al. (Bab, I., Passi-Even, L., Gazit, D., Sekeles, E., Ashton, B.A., Peylan-Ramu, N., Ziv, I., and Ulmansky, M.; Osteogenesis in in vivo diffusion chamber cultures of human marrow cells, Bone and Mineral 4; 373, 1988) observed bone in four of eight diffusion chambers implanted with human marrow from two child donors, however, these authors failed to observe bone when whole marrow from older donors was incubated in diffusion chambers in nude mice. In addition, Davies (Davies, J.E., Human bone marrow cells synthesize collagen, in diffusion chambers, implanted into the normal rat, Cell. Biol. Int. Rep. 11, 2: 125, 1987) did not observe bone formation in diffusion chambers inoculated with fresh marrow from a five year old female, nor was bone formation observed by Ashton, et al. (Ashton, B.A., Cave, F.A., Williamson, M., Sykes, B.C., Couch, M., and Poser, J.W.; Characterization of cells with high alkaline phosphates activity derived from human bone and marrow; preliminary assessment of their osteogenicity, Bone, 5:313-319, 1985) in diffusion chambers inoculated with cultured fibroblasts from composite pieces of bone and marrow from children and young adults.

In the present example, bone formation was not observed in diffusion chambers inoculated with cultured marrow-derived mesenchymal cells from several older donors. However, bone formation was observed in ceramic filled grafts with cultured marrow-derived mesenchymal cells from the same preparations of older donors (34-67 years old) which failed to generate bone in diffusion chambers. The factors which apparently make ceramics a more sensitive vehicle for bone differentiation from marrow-derived mesenchymal cells are unclear, but may involve direct accessibility of the marrow-derived mesenchymal cells to growth and nutrient factors supplied by the vasculature or direct interaction

with vascular cells which are limited because of diffusion chamber geometry (Jaroma, H.J., and Rotsila, V.A., Effect of diffusion chamber pore size on differentiation and proliferation of periosteal cells, Clin. Orthop., 236, 258, 1988) (Villanueva, J.E., and Nimni, M.E., Promotion of calvarial cell osteogenesis by endothelial cells in diffusion chambers, J. Cell. Biol., 109, 4, part. 2:42a (abstract).

The question of origin of the bone formed in the ceramic pores is important since the donor marrow-derived mesenchymal cells are not physically separated from the host cells as is the case for diffusion chambers. Recent data by Goshima, et al. (Jun Goshima, Victor M. Goldberg and Arnold I. Caplan, "The Origin of Bone Formed in Composite Grafts of Porous Calcium Phosphate Ceramic and Marrow Cells" (1989) Submitted) indicate that bone formation in ceramic grafts is a biphasic phenomenon with the initial bone formation being of donor origin. When this donor-derived bone has partially filled the pores of the ceramics, host-derived cells begin remodeling the donor bone, thus beginning the second phase of host-derived bone formation. Eventually, a marrow cavity forms in the center, with a cocoon of host-derived bone which has been laid on the partially remodeled inner surfaces of original donor bone. To confirm the origin of the bone formed with human marrow, the present inventors are currently assaying ceramic grafts with species-specific monoclonal antibodies directed against human osteocytes. The preliminary data shows antibody reactivity to the osteocytes within the grafts, thus suggesting that the bone formed in the porous ceramic is of human and not mouse origin.

Cultured marrow-derived mesenchymal cells originating from femoral head cancellous bone appear to be more osteogenic than cultured marrow-derived mesenchymal cells from iliac aspirated marrow; 9 out of 9 cancellous bone marrow samples produced bone in ceramics, whereas, 3 out of 4 aspirated marrow-derived mesenchymal cell samples produced bone in ceramics. In addition, bone was present in fewer pores in ceramics grafted with aspirated marrow-

derived mesenchymal cell than ceramics grafted with femoral head marrow-derived mesenchymal cells. The reasons for the differences is unclear, but, may be associated with the proximity of the harvested marrow stromal cells to the bone surface in the original tissue. Ashton, et al. (Ashton, B.A., Eaglesom, C.C., Bab, I., and Owen, M.E., Distribution of fibroblastic colony-forming cells in rabbit bone marrow and assay of their osteogenic potential by an in vivo diffusion chamber method, Calcif. Tissue Int., 36:83, 1984) showed that cultured rabbit marrow stromal cells differ in their colony forming potential in vitro and osteogenic potential in diffusion chambers depending on their original proximity to the endosteal surface. Cells closest to the endosteal surface were shown to have four times the colony forming efficiency as compared to cells of the core. In the present study, marrow from cancellous bone was harvested by vigorous vortexing to separate the cancellous bone from the marrow cells. This likely produces a population of marrow enriched in cells derived from near the endosteal surface, as compared to aspirate marrow where vigorous separation of marrow cells from cancellous bone is not possible. The inventors observed a consistently higher initial number of adherent cells from cancellous bone marrow as compared to aspirate marrow, which is similar to the observations of Ashton, et al. (Ashton, B.A., Eaglesom, C.C., Bab, I., and Owen, M.E., Distribution of fibroblastic colony-forming cells in rabbit bone marrow and assay of their osteogenic potential by an in vivo diffusion chamber method, Calcif. Tissue Int., 36:83, 1984).

In the case of marrow from adult donors, cartilage was not observed in this study or the study by Bab, et al. (Bab, I., Passi-Even, L., Gazit, D., Sekeles, E., Ashton, B.A., Peylan-Ramu, N., Ziv, I., and Ulmansky, M.; Osteogenesis in in vivo diffusion chamber cultures of human marrow cells, Bone and Mineral 4; 373, 1988). It may be that there is an age-dependent determination of marrow-derived cells for the osteogenic lineage over the chondrogenic lineage. Alternatively, culturing conditions in the

present study may be selective for osteoprogenitor cells over mesenchymal stem cells or may drive mesenchymal stem cells towards the osteogenic lineage prior to in vivo analysis in ceramics. Present studies are being directed towards addressing these possibilities.

The most important realization from the studies set forth in the above example is that the ceramics graft technique provides a sensitive assay for identifying the osteogenic potential of marrow-derived mesenchymal cells. Importantly, such osteogenic cells can be obtained from human donors of a wide age range. These observations indicate that the ex vivo expansion of cells possessing an osteogenic potential may be used for clinical circumstances requiring augmentation of osteogenesis.

EXAMPLE 2

Production of Cloned Hybridomas to Marrow-Derived Mesenchymal Cells

I. Immunization

A female mouse (CB6F/J Jackson Labs, Bar Harbor, Maine), approximately 14 weeks old at the beginning of the experiment, was immunized by peritoneal injections with cultured human marrow-derived mesenchymal cells obtained from multiple donors (see Table 3 below). The initial injection was followed by four booster injections spaced one week apart. Marrow cells from multiple donors were utilized for the purposes of producing monoclonal antibodies specific for common recognition sites on the mesenchymal stem cells.

TABLE 3

Background Information on Marrow Donors Used in Mouse Immunization

Prep. #	Date Obtained	Birth date	Type of Prep.	Clinical Diagnosis
H-20	12/16/88	04/09/37	Plug	DJD
H-21	01/05/89	01/01/38	Plug	DJD
H-23	01/12/89	02/23/09	Plug	DJD
H-27	01/17/89	12/12/25	Plug	DJD
H-31	01/19/89	Approx. 1929	Plug	DJD

Plug = a plug of cancellous bone and marrow which is scooped out of discarded femoral heads during hip transplant surgery

DJD = Degenerative joint disease

The Immunization Schedule

A. Day 0 - The initial injection consisted of cells from donor H-20 which were isolated and purified according to the procedure set forth above in Example 1. Cells from primary cultures were grown to confluence and replated (1:3). These first passaged cells were also grown to confluence and then released from the plate with 0.5 mM EGTA in Moscona for one hour. The cells were rinsed twice with Tyrodes and then reconstituted in 500 ul Tyrodes for injection into the mouse. The cells were dissociated to single cells by passing through a 20 gauge needle fitted on a one ml syringe and then injected into the peritoneal cavity of the mouse. Approximately 2.0 x 10^6 cells were used. All subsequent booster injections were prepared in a similar fashion.

B. Day 7 - Cells were derived from donor H-21 and H-23 and collected after confluence of the primary cultures. Approximately

equal numbers from each donor was used with the total cell count = 1.5×10^6.

C. Day 15 - Cells were derived from donor H-27 after primary culture. Total cell count = 1.0×10^6.

D. Day 22 - Cells were derived from donor H-31 at confluence after the first passage. Total cell count = 1.0×10^6.

E. Day 29 - Cells were derived from donor H-31. Cells were harvested at confluence after the first passage and cryopreserved in liquid nitrogen. Immediately prior to injection, cells were thawed and prepared and injected as described above on Day 0. Total cell count = 2.0×10^6.

At Day 25 blood was drawn from the tail vein of the mouse and serum was prepared by spinning out the blood cells. The serum was incubated to a frozen section of pelleted cultured human marrow cells and assayed by indirect immunofluorescence. The serum reacted positively to the cells in the frozen section, indicating that the immunization regiment had been successful in generating an immune response in the mouse to the cultured cells.

II. Fusion

After successfully completing the four week immunization process, the immunized mouse was sacrificed and its spleen cells were fused with SP 2/0 Myeloma cells obtained from Dr. Douglas Fambrough at the Carnegie Institute in Baltimore, Maryland, according to the specific process set forth below. However, in order to provide conditioned medium for the hybridoma cells produced during the fusion process, it was necessary to prepare at least a day prior to fusion a "feeder layer" comprised of conditioned medium and peritoneal macrophages. The peritoneal macrophages were added in order to clean the culture dishes through

phagocytic action. The following procedures were utilized in order to produce the feeder layer and to prepare the SP 2/0 Myeloma cells for fusion.

A. Preparation of Feeder Cells

Since lymphoid cells often grow poorly or die when grown at low density (the reasons for this are not well understood, but may relate to the requirements for "growth factors" or to the problems of toxic byproducts of the tissue culture vessel), the addition of a slow-growing or non-growing population of cells (usually termed "feeders") was necessary. The feeder cells utilized in the current process were produced according to the following procedure.

1. 0.34 M (11-64 g/100 ml) sucrose solution was filtered for sterilization and the solution was stored in 15 ml aliquots at 4°C.

2. A mouse was sacrificed by cervical dislocation and immersed completely in 70% EtOH. After several minutes in EtOH, the mouse was removed and laid supine on a styrofoam test tube rack.

3. A midline cut was made in the abdominal skin. Using blunt dissection, the skin was spread away from the peritoneum making sure not to puncture the thin membrane.

4. Using a 20 gauge needle, 5 ml of sucrose was injected into the peritoneum.

5. The needle was removed and the abdomen was gently massaged in order to liberate the macrophages. A 23 gauge needle was inserted into the abdomen and the 5 ml of sucrose was slowly recovered.

6. Steps 4 and 5 were repeated two times and the macrophages were pooled.

7. At 1000 rpm (200 g) the cells were spun for 5 minutes on a Sorvall GLC-4 with an H1000 rotor (r = 18.6 cm). The cell pellet was resuspended in 15 ml D-MEM (Dulbecco's Modified Eagle's Medium,

Gibco, Grand Island, NY, #430-2100)/10% FBS (Fetal Bovine Serum, Gibco, #430-2100) and spun.

8. The pellet was brought up in 2 ml of medium and the cells counted. An average yield was 3 to 6 million cells.

9. The cells were resuspended in Super HAT medium at a concentration of 1 x 10^5 cells/ml. In this regard, Super HAT medium consisted of 400 ml D-MEM, 120 ml FBS, 60 ml NCTC-109-Hybridoma Growth Supplement (M.A. Bioproducts #12-923A), 12 ml 50 X HAT or H+T (Boehringer Mannheim #644579 or #623091, respectively), 6 ml Solution I (supplemented D-MEM to provide proper pH and enhanced growth of the hybridomas, contained 0.2 units/ml Insulin, 0.5 mM Na Pyruvate, and 1.0 mM Oxaloacetic Acid), 6 ml L-Glutamine (Gibco #320-5030), 0.6 ml Gentamicin (M.A. Bioproducts #17-5182).

10. 0.1 ml of the suspension was then added to each well of ten 96-well culture dishes. Ten dishes were used per fusion.

11. The wells were stored in a 37°C humid incubator with 5% carbon dioxide, 95% air.

B. Preparation of SP 2/0 Myeloma Cells

The myeloma cells obtained from Dr. Fambrough were evaluated in order to ensure that they were growing well in culture for not less than 2 months prior to the fusion procedure. The cells were passed in D-MEM (Dulbecco's Modified Minimal Eagle's medium)/ 10% FBS/ 8-Azaguanine (Gibco, Grand Island, NY) in order to select for HAT sensitive cells. Passage of either myeloma or hybridoma cells was accomplished by serially diluting an aliquot across 12 wells of a 48-well plate.

Two weeks prior to fusion, the medium was step-wise adjusted up to 20% FBS. One week prior to fusion, several large flasks (75 cm^2, 20 ml of medium) of SP 2/0s were initiated. At that time two rows of a 24-well plate were seeded with SP 2/0s at low density. One day later, the medium was then changed in one of the rows with

the same Super HAT medium which was going to be used for fusion. (Prior to using any medium, a sample from each bottle was tested for sterility and bacterial growth). Within 24 hours, most of the cells fed HAT were dead, and by 72 hours, all cells fed HAT (hypoxanthine, aminopterin, thymidine) were dead. Cells fed SP 2/0 growth medium remained healthy.

Two days before the fusion, the SP 2/0s were split into three new flasks at various dilutions (i.e. 1:20, 1:10, 1:8, 1:4). The cells were carefully monitored at various dilutions and only those cells which were in log phase of growth were selected for fusion. There were no dead or dying cells in the selected population. A flask containing the good density of log phase cells contained a total of one to ten million cells.

C. <u>The Fusion Procedure</u>

<u>Materials</u> - per spleen

Dissecting scissors, large forceps, #5 dissecting forceps all in 70% EtOH

Frosted glass slides in 70% EtOH

One large watch glass (sterile) with cork stand

Several 100 mm Petri dishes

Ten 96-well culture dishes with feeder cells (prepared as indicated above)

SP 2/0 Myeloma cells (prepared as indicated above)

Water bath at 37°C

1 ml PEG 1500 at 37°C (Polyethylene Glycol, 2 gms in 75 mM HEPES (N-2-hydroxyethylpiperazine-N'-2 ethane sulphonic acid)

10 ml D-MEM/ 10% FBS/2X antibiotic

100 ml D-MEM (serum-free) on ice

25 ml D-MEM (serum-free) at 37°C

Two 50 ml plastic centrifuge tubes (sterile)

Hemocytometer

Super HAT at 37°C

4% Acetic acid

Cell Freezing Medium at 37°C

Method

1. Two frosted slides were removed from the EtOH and placed in a sterile Petri dish under a hood so that they would air-dry.

2. The mouse was sacrificed by cervical dislocation and immersed in 70% EtOH. After several minutes the mouse was placed on the large watch glass with its left side up.

3. A sterile 100 mm Petri dish with 5 ml D-MEM/10% FBS/2X antibiotic was set up for receiving the spleen.

4. The skin and then the abdominal wall were opened on the left upper quadrant of the mouse.

5. The spleen was removed and placed in the Petri dish. Using the dissecting forceps, as much of the white fibrous connective tissue as possible was removed. The cleaned spleen was placed into another Petri dish with D-MEM/10% FBS/2X antibiotic.

6. Any residual EtOH was flame dried from the glass slides. The slides were then used to slice the spleen into three pieces. Each of these pieces was then gently ground between the frosted ends of the slides to liberate the spleen cells. The cell suspension was then aspirated with a pipet, large pieces of connective tissue sank immediately to the tip. These were then discarded and the remaining cell suspension was transferred into a sterile 50 ml centrifuge tube.

7. Another 5 ml of the D-MEM/10% FBS/2X antibiotic was added to the Petri dish and this was used to the collect residual cells. The solution was pipeted as above, the cells added into a 50 ml centrifuge tube, and placed on ice.

8. The SP 2/0 myeloma cells were collected by centrifugation in a sterile 50 ml centrifuge tube. At the same time, the spleen cells were pelleted at 1000 rpm for 5 minutes.

9. Both sets of cells were washed three times with 5 ml of cold, serum-free D-MEM (i.e. serum interferes with the fusion of these two cell types).

10. Both sets of cells were then resuspended in 5 ml of ice cold serum-free D-MEM by gently tapping and pipetting. These cell suspensions were kept cold on ice.

11. The spleen cells were then counted using the hemocytometer. A 30 μl aliquot of cells was taken and added to 30 μl of 4% acetic acid. The acid lysised the RBCs and made it easier to count the splenocytes. The sample was applied to the hemocytometer and the cells were counted in 25 large squares. This number, X, when divided by 50 gave the concentration of cells, in millions per ml. There was approximately 100 million spleen cells total. Only ten million spleen cells were used in the fusion. The rest were frozen. These cells were then thawed, washed twice with serum-free D-MEM, and used for another fusion. For this reason, the cells were frozen in aliquots of ten to twenty million cells per vial.

12. The fusion process was performed using a 3:1 ratio of spleen cells to SP 2/0s (i.e. the SP 2/0s were counted and approximately 3 million cells were used, this gave about 700-800 growing wells out of the 960 initially seeded). The SP 2/0 cell suspension was then added to the spleen cells and the mixture was spun for 5 minutes at 1000 rpm. All of the supernatant was removed thereby leaving the pellet as dry as possible. The pellet was loosened by tapping the tube briskly. It was important to break up this pellet so that the PEG would contact as many cells as possible.

13. The tube was then placed in a 37°C water bath. 1 ml of 37°C PEG solution (the fusion promoter) was added drop wise directly onto the pellet over a full one minute period. The tube was rotated while the PEG was added. To suspend the cells, the tube was gently swirled. The mixture was then allowed to sit in the water bath for about one minute.

14. While rotating the tube 1 ml of 37°C serum-free D-MEM was added over a one minute period.

15. 20 ml of 37°C serum-free D-MEM was then added over a four minute period.

16. At 1000 rpm the mixture was spun for five minutes. The supernatant was immediately removed and the pellet was resuspended in 100 ml Super HAT medium. 0.1 ml was placed in each well of the ten plates containing the macrophages.

Feeding Schedule

The cultures were fed after fusion through the replacement of a portion of the culture medium with fresh medium. As a result, the feeding gradually diluted out any of the antibody made by normal (unfused) antibody-secreting spleen cells while also removing waste product and replenishing the nutrients.

1. After 3 days 0.1 ml of medium was removed from each well taking care not to disturb the cell layer and replaced with fresh Super HAT.

2. The medium was charged again at 5 days. Most of the cells (i.e. unfused spleen cells and unfused myeloma cells) were dead or dying at this stage. After a week in Super HAT medium, it was assumed that all of the parental myeloma or spleen cells were dead, and any growing cells were hybrids. The macrophages cleared much of the debris present in the medium. If the medium turned yellow and there were no colonies of hybridoma cells observed, the medium was charged again at 7 days. If the medium was yellow and there were colonies of growing cells observed, the medium was screened for the presence of antibody.

3. By 7 to 10 days after fusion, beautiful colonies of hybridoma cells were apparent. They were observed growing on the periphery of each well. Out of the 960 wells plated, 764 wells exhibited hybridomas.

4. The culture supernatant (50 μl) was then removed for screening when 1/4 to 1/3 of each well was covered with growing cells.

III. Preliminary Screens Prior to Cloning

There were two general screening methods used to characterize the antibodies that were secreted by the various hybridomas: the Enzyme-Linked Immunosorbent Assay (or ELISA Assay), and the Indirect Immunofluorescence Assay. What follows is a summary of how these assays were used to screen the hybridoma colonies present in the 764 wells to identify those hybridomas which produce antibodies with the specificity for the cultured marrow mesenchymal stem cells.

A. IgG ELISA - When the colonies initially became visible the culture supernatant was screened on ELISA to goat anti-mouse IgG. This screen was set up to identify any colony composed of hybridomas which secrete antibodies with the IgG isotype. Since this isotype is the major secretary antibody and is also the easiest to purify and use, it was preferred and screened for over IgM and IgA isotopes which may also contain specificity for the desired epitope.

Detailed Protocol for Performing the Goat anti-mouse IgG ELISA Assay

Preparation of goat-antimouse IgG ELISA plates

1. 500 ul of goat-antimouse IgG (Organon Teknika, Catalog # 06110081) was diluted in 100 ml of Dulbecco' PBS (D-PBS) (GIBCO) and 50 ul were added to each well of 96 well vinyl microtitration plates (GIBCO).

2. Antibody was allowed to incubate for one hour at room temperature.

3. Plates were then rinsed twice with D-PBS containing 1% bovine serum albumin (BSA), and then incubated with D-PBS containing 1% BSA for one hour to block non-specific protein binding sites.

4. Plates were rinsed once with D-PBS containing 0.1% BSA, sealed in plastic bags and stored at 4°C until use.

Running of ELISA Assay

1. 50 ul aliquots of antibody culture supernatants were added to wells on the ELISA plates and allowed to incubate in a humidified chamber for one hour at room temperature.

2. Plates were rinsed four times with Tris-buffered saline (TBS) containing 0.1% BSA.

3. Alkaline phosphatase-conjugated goat antibody specific for mouse IgG, IgM, and IgA (Organon Teknika, Catalog # 86110231) was diluted 1:100 - 1:250 in TBS with 0.1% BSA and 50 ul was added to each well for one hour at room temperature in a humidified chamber.

4. Plates were rinsed four times with TBS with 0.1% BSA.

5. .0093 g of Phosphatase Substrate (Sigma) was dissolved in 10 ml of Substrate buffer which was composed of 50 mM glycine and 1 mM $MgCl_2$ pH 10.5. 50 ul was added to each well and allowed to incubate at 37°C for one hour. A yellow color in the wells was interpreted as positive antibody reactivity.

6. Positive and negative controls consisted of identical analysis of immunized mouse serum and culture medium, respectively.

The results of the assays indicated that out of the 764 wells which exhibited hybridoma growth, only 245 of these wells tested positive for the secretion of antibodies with the IgG isotype. This is demonstrated in the photograph of the ELISA assay results set forth in Figure 5.

B. Frozen Sections of Pelleted Cells by Indirect Immunofluorescence - Culture supernatant from colonies which screen positive for IgG were then screened against frozen sections of pelleted cultured marrow-derived mesenchymal cells by indirect immunofluorescence. This assay was designed to identify antibodies which bonded to epitopes on the cultured marrow-derived mesenchymal cells.

Detailed Protocol of Antibody Binding to Pelleted Cultured Human Marrow Cells by Indirect Immunofluorescence Assay

Preparation of Frozen Sections of Culture-expanded Human Marrow-derived Mesenchymal Cells

1. 4 ml of 0.25% Trypsin with 1 mM EDTA (GIBCO) was added to confluent cultures of human marrow-derived mesenchymal cells in 100 mm culture dishes and allowed to incubate at 37°C for five minutes.

2. The enzymatic activity of the trypsin was stopped by addition of 2 ml of calf serum. The cells were pelleted by centrifugation at 1000 x g, and the supernatant was discarded. The pelleted cells were rinsed two times with PBS.

3. After the second rinse the supernatant was removed and the cell pellet was dispersed into a small cup containing OCT Tissue Tek Compound (Miles Inc.) and frozen in liquid nitrogen. Frozen blocks of cells were stored at 0°C in plastic bags until sectioned.

4. The blocks of tissue were sectioned at 6 microns/section and placed on gelatin coated slides. Slides were stored at 0°C in slide boxes until needed.

Indirect immunofluorescent staining

1. Slides were removed from the freezer and allowed to warm to room temperature before using.

2. The sections were covered with 50-100 ul of antibody culture supernatant and incubated at room temperature for one hour

in a humidified chamber. The slides were rinsed four times with 0.1% BSA-PBS.

3. The sections were then covered with 50 ul of FITC-conjugated Goat-antimouse IgG (Organon Teknika, Catalog #1211-0231) which had been diluted 1:100 to 1:200 with 0.1% BSA-PBS and were allowed to incubate for one hour at room temperature in a humidified chamber.

4. The slides were rinsed four times with 0.1% BSA-PBS and coverslipped with a drop of PPD immunofluorescence mounting medium, and observed with an Olympus BH-2 epi-fluoresence microscope.

5. Negative control slides consisted of identical analysis of cells with culture medium which did not contain antibody.

The test results identified those antibodies which bonded to epitopes on the culture marrow-derived mesenchymal cells. See Figures 6A-6H which are photomicrographs of typical frozen sections of pelleted culture-expanded human marrow derived cells. In addition, close observation of the staining pattern gave an indication of the cellular location of the antigen; intracellular, cell surface, or both. Of interest were only the antibodies which reacted to the cell surface, however since interpretation of the immunofluorescence pattern for intracellular or cell surface was not 100% accurate, all of the antibodies which react to any part of the cultured marrow cells were kept, and those antibodies which gave a negative response were screened out. Out of the 245 wells which tested positive for IgG secretion, 171 of these wells tested positive to frozen sections of pelleted cultured marrow cells.

C. Indirect Immunofluorescence to Live Cells in Micromass Cultures - Cultured human marrow-derived mesenchymal cells were replated into a small mass on the center of a tissue culture dish. This type of culture is referred to as a micromass. The cells normally remain viable, spread and replicate in these masses. Hybridoma supernatants which indicated positive reactivity to the

screens in parts A and B above were incubated with the cells in these micromass cultures and the reactivity was measured by indirect immunofluorescence according to the protocol set forth below. Since the cells being analyzed in this assay were living cells, this assay identified antibodies which bonded only to the cell surface of the cell and gave negative results to antibodies which bonded to intracellular epitopes.

Detailed Protocol of Antibody Binding to Living Culture-expanded Marrow-derived Cells in Micromass

Preparation of the micromass cultures

1. Cells from confluent marrow-derived mesenchymal cell cultures were released with 0.25% trypsin containing 1 mM EDTA as described above. After stopping the activity of the trypsin with calf serum, the cells were pelleted by centrifugation and the supernatant was removed.

2. Cells were rinsed once with 5-10 ml BGJ_b Complete medium and then were resuspended in Complete medium at a concentration of 500,000 cells per ml. 50 ul of cell suspension was transferred to the center of a 35 mm tissue culture dish (Falcon) and incubated overnight at 37°C.

Indirect immunofluorescent staining

1. The 35 mm dishes were rinsed three times with PBS. 100 ul aliquots of antibody culture supernatants were added to each dish and incubated for one hour at room temperature in a humidified chamber.

2. Dishes were rinsed three times with 0.1% BSA-PBS, then 100 ul of FITC-conjugated Goat antibody specific for mouse IgG was added to each plate after being diluted 1:100 to 1:200 with 0.1% BSA-PBS. Dishes were incubated for one hour at room temperature in a humidified chamber.

3. Dishes were rinsed three times with 0.1% BSA-PBS and coverslipped after applying a drop of PPD immunofluorescent

mounting medium. Immunofluorescent staining was observed using an Olympus BH-2 epi-fluoresence microscope.

Utilizing the above described protocol, out of the 171 wells which tested positive both for IgG secretion and the specificity to frozen pelleted cultured marrow cells, only 15 of these wells tested positive to specificity to living cultured marrow-derived mesenchymal cells. See the photomicrographs set forth in Figure 7A-7H which demonstrate the typical results of an indirect immunofluorescence analysis of living cultured marrow-derived mesenchymal cells in micromass.

IV. <u>Cloning by Limited Dilution of Selected Colonies</u>

The hybridomas which tested positive to each of the three preliminary screens (i.e. the 15 wells) were cloned by the following process in order to ensure that the subsequent screening steps were preformed on hybridomas which originated from a single clone. Since multiple cell lines are usually present in the original "parent" well, cloning of the cells was required to obtain a monoclonal hybridoma cell line. By replating cells at a density of less than one cell per well, any colony which grew in a well should be the progeny from that cell. When all the growing wells contained the antibody of interest for two serial clonings, the cell line was considered to be monoclonal. In addition, the cloning by limited dilution was also performed in order to reduce the risk of overgrowth by non-producer cells.

<u>Preparation of Feeder Layer Cells and Conditioned Medium</u>

1. Mouse splenocytes were used instead of macrophages at this stage. Although the splenocytes were easier to collect, they added to the debris in the wells whereas the macrophages actually cleared the debris.

2. A mouse was sacrificed and its spleen was removed as described above.

3. The cells were liberated as previously described, counted, and added to 37°C Super HAT so that their concentration was 1 million per ml.

4. When 96 well plates were used for plating, 0.1 ml of this suspension was added to each well. When 24 well plates were used for plating, 0.5 ml of this suspension was added to each well.

5. Alternatively, this cell suspension was also grown in large culture flasks (20 ml) for up to three days. During those three days, the suspension was used to create feeder layers. If the cells were not used within the three days, the medium was collected by passing the suspension through a sterile 0.22 μm filter. This medium, which can be stored at 4°C for several weeks, was a useful additive for poorly growing cultures. However, this medium was not used instead of feeder layers.

<u>Cloning</u>

1. In all of the cases below, the cells were plated into wells already containing a feeder layer (see above).

2. The cells and medium were transferred from the selected parent wells of a 96-well plate to one well in a 24-well plate.

3. 200 μl of medium was added back to the parent wells so that residual parent cells could continue to grow. The parent wells served as back-ups in case of error.

4. Cell growth was monitored in the 24-well plate. It generally took about 3 to 7 days for a growing colony to cover half the well. In those wells which exhibited slow growth, additional conditioned medium was added.

5. When the cells were growing nicely, the medium was again screened for antibody. Since there was no point in cloning these cells if they had stopped secreting antibody, this was a necessary step.

6. If the antibody of interest was present, a small aliquot (50 μl) of cells was removed from the edge of the colony. This is normally where the most actively growing cells are found.

7. 10 μl of this aliquot was added to 30 μl of 0.5% Trypan blue dye. The viable cells were counted, the dead cells appeared blue. It was important to account for the dilution with Trypan when the final cell concentration was determined.

8. The remaining 40 μl aliquot was manipulated so that 100 cells were removed with certainty.

9. These 100 cells were added to 20 ml of Super HAT, the suspension was mixed and evenly plated into two 96-well plates. This resulted in two plates of cells at 0.5 cells per well.

10. Colonies developed in about half of the wells within 5 to 10 days. The fast growing colonies were screened for antibody. When positive colonies were found, four of the colonies were transferred to new wells on a 24-well plate.

11. 200 μl of medium was added back to the 96-well plate, and the new 24-well plate was treated exactly as the original 24-well plate above. This provided another source of back-up cells if cloning (or anything else) fails. Approximately 3 million cells were frozen per vial.

12. Approximately two weeks after the fusion, the cells were switched from Super HAT to Super H+T medium.

13. Cloning was continued using the above techniques until 100% of the subclones were positive for two generations.

14. The successively more "clonal" cells were also frozen for precautionary measures.

15. Once it was satisfied that the cell line was monoclonal, the culture medium was changed to Hybridoma 20%. This medium, which allows for enhanced cell growth, consisted of 78 ml D-MEM, 20 ml FBS, 1 ml Glutamine (Gibco #320-5030), 1 ml Solution I (see above), 0.1 ml Gentamicin (M.A. Bioproducts #17-5187).

V. Recovering the Monoclonal Antibodies Secreted by the Hybridomas

The monoclonal antibodies secreted by the cloned hybridomas were recovered by either culturing the cloned hybridomas (individually) in the Hybridoma 20% medium and recovering the antibody from the medium or culturing the cloned hybridomas intraperitoneally in mice and harvesting the antibody containing the malignant ascites or serum from the mice. In this regard, through the use of the following procedure, hybridoma cells grown in the peritoneal cavity of the mice produced an ascitic fluid rich in monoclonal antibodies.

1. The recipient mouse was "primed" 5 to 10 days prior to tumor cell inoculation by intraperitoneally injecting each mouse with 0.5 ml of Pristane (2, 6, 10, 14, Tetramethylpentadecane; Sigma, Catalog #T-7640). The mice which were used to grow the tumors were of the same strain as that used to initially generate the hybridoma cell lines. The mice used were approximately 8 to 12 weeks of age. One tumor line was grown in 6 mice at a time.

2. In culture, the established monoclonal cell line was grown in log phase. Approximately 30 million cells were collected.

3. The cells were spun at 1000 rpm for five minutes and then removed from the culture supernatant.

4. Since the serum interferes with tumor production and contaminates ascites fluid, the cells were washed in serum-free medium and spun again.

5. The cells were resuspended in serum-free medium (i.e. DMEM-HG, Gibco, Grand Island, NY) such that the final cell density was 10 million per ml.

6. Using a 23 gauge needle, 0.5 ml (5 million cells) of the suspension were injected into the peritoneum of each recipient mouse.

7. Approximately one week later, the mice appeared to have "bloated" abdominal cavities. Once they appeared to have enlarged

to about the size of a plum, the ascites fluid present in the peritoneal cavity was tapped.

8. During tapping the mice were lightly anesthetized using Metafane (Methoxyflurane; Pitman-Moore, Inc., Catalog #NDC 11716-5943-4). Using a 22 gauge needle, the abdominal skin was pierced so that the needle was very superficially within the peritoneum. The ascites fluid then dripped out of the needle and into an awaiting vessel. By moving the mouse and/or the needle, it was possible to get as much as 5 mls of fluid. This fluid yielded antibody in the range of 0.5 to 5 mg/ml.

9. The animals were placed back in their cages for recovery and further tapping.

10. In the animals which did not recover from anesthesia at any stage of the experiment, the residual ascites fluid was obtained by surgically opening the abdomen and removing pooled fluid. In this regard, it was important that the fluid was not collected from mice which had been dead for more than one hour.

11. Once the fluid was collected, it was spun at 3000 rpm for 5 minutes to pellet out the RBCs and other undesirable cells.

12. Sodium azide was then added so that its final concentration was 0.02%. This ascitic fluid was then stored in small aliquots at -70°C. The stability of each antibody to freezing and thawing was tested before the entire ascites prep was frozen.

VI. Screening of Cloned Hybridomas

The cloned hybridomas (15 wells) were screened against a series of mesenchymal and non-mesenchymal derived tissues to identify the degree of specificity of the monoclonal antibodies to the cultured marrow-derived mesenchymal cells.

The test results of the three optimal hybridomas, i.e. SH2, SH3, and SH4 were evaluated against a control for background noise. The results are set forth below in Table 4. Along this line, in Table 4, (-) indicates no visible reactivity above the control; (±)

indicates less than 1% reactivity above the control and for evaluation purposes was taken to be (-); and, (+) indicates substantial reactivity above the control. The negative control in each experiment involved incubating the cells or tissue of interest with SB-1, a monoclonal antibody to chicken alkaline phosphatase which does not react with human cells (without antibody), followed by rinsing the incubation with FITC-labelled second antibody.

A. The first level and most important level of specificity was that the monoclonal antibodies did not react to the hemopoietic lineage cells in marrow. To determine this, whole marrow and several partial fractionations of marrow were screened against hybridoma culture supernatant by indirect immunofluorescence according to the protocol set forth below. With fluorochrome isothiocyanate (FITC), the positive cells, and the percent positive cells, were noted, however, all hybridomas were kept for screening at the next level.

Preparation of marrow

Whole marrow and low density Percoll Fractions were prepared as described above in Example 1 and processed for frozen sections as described above for culture-expanded marrow-derived mesenchymal cells.

Indirect immunofluorescence staining

Sections of whole marrow and low density percoll fractions were screened with antibody culture supernatant from cloned by hybridomas using the same procedure as described above for screening culture expanded mesenchymal cells in frozen sections.

B. Screening of Monoclonal Antibodies Against Mesenchymal derived Tissue - A variety of mesenchymal tissues were obtained at surgery or autopsy to determine if the monoclonal antibodies reacted to epitopes common to marrow-derived mesenchymal cells and differentiated mesenchymal tissues. Frozen sections of the tissues

were screened against hybridoma culture supernatant and analyzed by indirect immunofluorescence according to the following procedure.

Detailed Protocol for Screening Tissue Section to Cloned Hybridoma Supernatants by Indirect Immunofluorescence

Preparation of the tissue section

1. The following tissues were obtained as surgical biopsies or at autopsy from human patients: skin, foreskin, intestine, heart, skeletal muscle, lung, liver, brain, tendon, ligament, gall bladder, articular cartilage, femur, rib cartilage, and periosteum.

2. The following animal tissues were also obtained for screening: chicken bone and marrow, rabbit bone and marrow, rat bone, and bovine cartilage and bone.

3. The tissues were each embedded in OCT-Tissue Tek Freezing Medium (Miles Inc.) and frozen into blocks in liquid nitrogen and stored at 0°C until use.

4. The tissue blocks were sectioned at 6 microns and placed on gelatine coated slides and stored at 0°C until use.

Indirect immunofluorescent staining

Tissue sections were screened against hybridoma culture supernatant from cloned hybridomas using the same procedure as described above for screening culture-expanded cells in frozen sections.

The positive and negative reactivities were noted and the patterns of reactivity described (Table 4). All cloned hybridomas were kept for screening at the next level.

C. Screening of Monoclonal Antibodies Against Non-mesenchymal derived Tissues - The overall objective of this screening protocol was to identify hybridomas which secreted antibodies which were specific for marrow-derived mesenchymal cells

and/or their lineage descendants. An antibody which reacted to non-mesenchymal derived tissue was therefore not as specific or unique. Many non-mesenchymal derived tissues were obtained at autopsy and frozen sections are prepared. Hybridoma culture supernatant was incubated with these sections and antibody reactivity was analyzed by indirect immunofluorescence as described above. The positive and negative reactivity was identified as well as the pattern of reactivity.

TABLE 4

Reactivity of Optimal Hybridomas and Associated Monoclonal Antibodies with Various Tissue Cells

		Monoclonal Antibodies		
		SH2	SH3	SH4
A.	Preliminary Screens			
1.	IgG-ELISA	+	+	+
2.	Pelleted Cultured Cells	+	+	+
3.	Micromass of Cultured Cells	+	+	+
B.	Marrow Hemopoietic Cells			
1.	Fresh Whole Marrow	-	±[a]	±[a]
2.	Low Density Percoll Fraction	±[a]	-	-
C.	Mesenchymal-Derived Tissues			
1.	Femoral Head (bone) (HCl)	-	-	-
2.	Rib bone and marrow (RDO)	±[b]	-	-
3.	Rib cartilage	-	+	-
4.	Skeletal muscle	-	-	-
5.	Cultured Periosteal Cells	-	+	+
6.	Cultured Periosteal Cells (micromass)	-	+	+
7.	Ligament	±[b]	±[b]	-
8.	Tendon	-	-	±[b]
9.	Articular Cartilage	-	+	+
10.	Femoral Shaft (RDO, HCl)	-	-	-
D.	Non-Mesenchymal Derived Tissues			

1.	Foreskin	-	-	-
2.	Breast skin	-	-	-
3.	Intestines	±[b]	-	-
4.	Heart	±[b]	-	-
5.	Lung	±[b]	-	-
6.	Liver	±[b]	±[b]	-
7.	Brain	-	-	-
8.	Gall Bladder	-	-	+

[a] less than 1% over control
[b] reactivity to tissue matrix not associated with cells

The above results clearly indicate that three hybridomas, i.e. SH2, SH3, and SH4 have been identified and cloned. These hybridomas are useful for the analysis of marrow-derived mesenchymal cells. All three of the hybridomas secrete antibodies which react with the cell surface epitopes on 99-100% of cells in assays of culturally expanded marrow-derived mesenchymal cells. In contrast, each of the three hybridomas secrete antibodies which react with less than 1% of the cells in assays of whole marrow. The ability of these antibodies to selectively bind marrow-derived mesenchymal cells and not hemopoietic cells makes them excellent probes for quantitating the number of marrow-derived mesenchymal cells in samples of marrow as well as for purifying such cells from marrow.

In addition, all three of the hybridomas showed mostly negative cross-reactivity when screened against a variety of mesenchymal and non-mesenchymal derived tissues, although some cross reactivity was observed with each. Of particular interest, SH3 and SH4 cross-reacted to cell surface determinants on cultured cells derived from human periosteum. Since the inventors have previously shown periosteum to be another source of marrow-derived mesenchymal cells, the cross reactivity of the above antibodies to cell surface epitopes on periosteal cells suggests a structural relationship between the marrow-derived mesenchymal stem cells and periosteum-derived mesenchymal stem cells. The SH2 antibody,

however, did not react to periosteum-derived mesenchymal stem cells even though it did bind to marrow-derived mesenchymal stem cells. This selectivity makes the SH2 antibody useful for distinguishing between marrow-derived and periosteum-derived mesenchymal stem cells. The selectivity of the SH2 antibody coupled with the cross reactivity of SH3 and SH4 suggest that marrow derived and periosteum derived mesenchymal stem cells are related but not identical.

EXAMPLE 3

The monoclonal antibodies produced above can also be utilized to distinguish between marrow-derived mesenchymal cells and marrow-derived hemopoietic cells. The ability to make such a distinction is useful for developing diagnostic reagents which quantitate the number of mesenchymal cells in samples of whole marrow. As a result, diagnostic reagents containing the monoclonal antibodies may then be used to identify patients with abnormally low mesenchymal cell numbers. A low level of mesenchymal cells may prove to be an indicator of abnormally low bone synthesis, which leads to osteoporosis.

In addition, monoclonal antibody-based diagnostic techniques are also useful for measuring the mesenchymal cell concentration in marrow harvested for future bone marrow transplanation. A low level of mesenchymal cells indicates a decreased probability of successful bone marrow transplantation because the marrow stroma will not develop quickly and completely without adequate mesenchymal cells to differentiate into stromal cells. In addition, assays which distinguish between marrow-derived mesenchymal cells and marrow-derived hemopoietic cells can also be used to purify mesenchymal cells from whole marrow. The purified cells may then be culture-expanded more efficiently and used to augment skeletal repair of bone marrow transplantation.

A procedure design to demonstrate the effectiveness of the monoclonal antibodies, such as the SH2 mesenchymal monoclonal

antibody produced above, to distinguish between marrow-derived mesenchymal cells and marrow hemopoeitic cells would include the following generally defined processes:

(1) Indirect immunofluorescent staining to cultured mesenchymal cells and whole marrow cells in suspension or solution.

(2) Indirect immunofluorescent staining of cryosections of culture-expanded mesenchymal cells and cryosections of whole marrow cells.

(3) Flow cytometric quantitation (or purification) of indirect immunofluorescent-stained culture-expanded mesenchymal cells mixed in a suspension of whole marrow cells.

Furthermore, the monoclonal antibodies produced above can also be utilized to distinguish between marrow-derived mesenchymal cells and periosteal-derived mesenchymal cells. Such a distinction would be useful for developing a diagnostic assay to quantitate marrow-derived mesenchymal cell numbers in situations were both types of mesenchymal cells might be present. For example, this type of assay would be useful in determining the relative contribution of marrow derived mesenchymal cells or periosteal-derived mesenchymal cells to the healing process after various types of fractures.

A procedure design to demonstrate the effectiveness of the monoclonal antibodies, such as the SH2 monoclonal antibody, to distinguish between marrow-derived mesenchymal cells and periosteal-derived mesenchymal cells would include the following generally defined processes:

(1) Indirect immunofluorescent staining of culture-expanded marrow-derived mesenchymal cells from a

suspension which also contains culture-expanded periosteal-derived mesenchymal cells using the SH2 monoclonal antibody.

(2) Indirect immunofluorescent staining of cryosections of a mixed population of known amounts of culture-expanded marrow-derived mesenchymal cells and culture-expanded periosteal-derived mesenchymal cells.

(3) Indirect immunofluorescent staining of living culture expanded marrow-derived mesenchymal cells and living cultured-expanded periosteal-derived mesenchymal cells in culture (in the form of micromass).

The invention has been described with reference to the preferred embodiment. Obviously, modifications and alterations will occur to others upon reading and understanding the preceding detailed description. It is intended that the invention be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.

Having thus described the preferred embodiment, the invention is now claimed to be:

1. A hybridoma which produces monoclonal antibodies which recognize an antigen on the cell surface of progenitor marrow-derived mesenchymal cells but do not recognize an antigen on hemopoietic cells.

2. A monoclonal antibody which recognizes an antigen on the cell surface of progenitor marrow-derived mesenchymal cells but does not recognize an antigen on hemopoietic cells.

3. A hybridoma which produces antibodies which react with mesenchymal derived tissues and fails to react with hemopoietic cells and non-mesenchymal derived tissues.

4. A monoclonal antibody which reacts with mesenchymal derived tissues and fails to react with hemopoietic cells and non-mesenchymal derived tissues.

5. A hybridoma which produces monoclonal antibodies which react with an epitope on the cell surface of a human marrow-derived mesenchymal cell and fails to react with an epitope on a human hemopoietic cell as shown by indirect immunofluorescence.

6. A monoclonal antibody which reacts with an epitope on the cell surface of a human marrow-derived mesenchymal cell and fails to react with an epitope on a human hemopoietic cell as shown by indirect immunofluorescence.

7. A hybridoma which secretes monoclonal antibodies which react with the cell surface of a progenitor mesenchymal cell and do not cross-react with a hemopoietic cell selected from the group consisting of ATCC HB 10743, ATCC HB 10744, and ATCC HB 10745.

8. The monoclonal antibodies produced by the hybridomas of claim 7.

9. The monoclonal antibodies that bind specifically to mesenchymal stem cells.

10. A hybridoma having the identifying characteristics of ATCC HB 10743.

11. A hybridoma having the identifying characteristics of ATCC HB 10744.

12. A hybridoma having the identifying characteristics of ATCC HB 10745.

13. A monoclonal antibody produced by hybridoma ATCC HB 10743.

14. A monoclonal antibody produced by hybridoma ATCC HB 10744.

15. A monoclonal antibody produced by hybridoma ATCC HB 10745.

16. A method for preparing monoclonal antibodies which are specific for mesenchymal stem cells comprising the following steps:

a) immunizing mice with purified mesenchymal stem cells;

b) removing the spleen from said mice and preparing a suspension of spleen cells;

c) fusing said spleen cells with mouse myeloma cells;

d) diluting and culturing the fused cells in a selective medium which essentially only facilitates growth of fused spleen-myeloma cells (the hybridoma cells);

e) evaluating the culture supernatant for the desired antibody activity;

f) inoculating single cultures with cells from cultures producing the desired antibodies;

g) cloning the positive hybridoma cells; and,

h) recovering the antibodies from the culture supernatant.

17. A method of producing a hybridoma which secretes monoclonal antibodies which are specific to the cell surface of mesenchymal stem cells and do not react with hemopoietic cells, which comprises:

a) immunizing a mouse with purified mesenchymal stem cells through at least one intraperitoneal injection of said mesenchymal stem cells;

b) removing the spleen from said immunized mouse and making a suspension of the spleen cells;

c) fusing the spleen cells, in the presence of a fusion promotor, with myeloma cells to form hybridomas capable of producing monoclonal antibodies;

d) growing the hybridomas in a medium which will support growth of said hybridomas so that the monoclonal antibodies are secreted into the medium;

e) preliminarily screening the medium containing the hybridomas and the secreted antibodies in order to characterize the antibodies as follows:

(i) screening the medium to anti-mouse IgG to identify hybridomas which secrete antibodies with the IgG isotype;

(ii) screening the medium which reacted positively for IgG against frozen sections of marrow mesenchymal stem cells to identify antibodies which bind to either the intracellular or the cell surface epitopes on the marrow mesenchymal stem cells; and,

(iii) screening the medium which reacted positively to the IgG and the frozen sections of marrow mesenchymal stem cells with live marrow mesenchymal stem cells in order to identify antibodies which bind only to the cell surface epitopes of the mesenchymal stem cells;

f) individually culturing the hybridomas present in the medium which tested positive for antibodies that bind only to the cell surface of the mesenchymal stem cells by replating the hybridomas at a density of less than one hybridoma per well and allowing for said individually plated hybridomas to expand and secrete monoclonal antibodies in said medium;

g) screening the medium containing the monoclonal antibodies secreted from each hybridoma against a series of mesenchymal and non-mesenchymal derived tissues in order to identify the degree of specificity of the antibodies, wherein said screening process comprises the steps of:

(i) reacting said medium with marrow hemopoietic cells in order to determine the monoclonal antibodies which failed to react with said hemopoietic cells;

(ii) reacting said medium with mesenchymal derived tissue in order to determine if the monoclonal antibodies reacted to epitopes common to mesenchymal stem cells and differentiated mesenchymal tissue;

(iii) reacting said medium with non-mesenchymal derived tissue in order to determine the monoclonal antibodies which are not specific for mesenchymal stem cells or their lineage characteristics;

h) selecting said antibody containing medium in which the monoclonal antibodies are specific to the cell surface of mesenchymal stem cells or their lineage descendants and do not react with hemopoietic cells; and,

i) cloning the hybridoma which secretes said monoclonal antibodies.

18. The hybridoma produced according to the method of claim 17.

19. The method of claim 18, wherein said purified mesenchymal stem cells are human mesenchymal stem cells purified according to the process of Example 1.

20. The method of claim 18, wherein said purified mesenchymal stem cells are culturally expanded purified mesenchymal stem cells.

21. The method of claim 18, wherein said purified mesenchymal stem cells are culturally expanded purified human mesenchymal stem cells.

22. The method of claim 18, wherein at least 1.0×10^6 culturally expanded mesenchymal stem cells are interperitoneally injected into said mouse.

23. The method of claim 18, wherein said myeloma cells are SP 2/0 myeloma cells.

24. The method of claim 18, wherein said hybridoma is cultured in a histocompatible animal and said antibodies are recovered from the ascitres fluid of said animal.

25. A method of producing a hybridoma which secretes a monoclonal antibody which is specific to the cell surface of a mesenchymal stem cell and does not react with a hemopoietic cell, comprising the steps of:

a) immunizing a mouse with purified mesenchymal stem cells through intraperitoneal injections of at least 1.0×10^6 dissociated mesenchymal stem cells one week apart for at least four consecutive weeks;

b) removing the spleen from said mouse on completion of the immunization process and making a suspension of the spleen cells; and,

c) fusing said spleen cells in the presence of a fusion promoter with mouse myeloma cells to form a hybridoma capable of producing monoclonal antibodies.

26. The hybridoma produced according to the method of claim 25.

27. The method of claim 25, wherein the purified mesenchymal stem cells are culturally expanded.

28. The method of claim 25, wherein the purified mesenchymal stem cells utilized for the intraperitoneal immunization injections are derived from different sources.

29. The method of claim 25, wherein the purified mesenchymal stem cells are culturally expanded purified human marrow-derived mesenchymal cells.

30. The method of claim 25, wherein said mouse myeloma cells are SP 2/0 myeloma cells.

31. A method for determining the presence of mesenchymal stem cells in a sample comprising the steps of:

a) preparing monoclonal antibodies which bind specifically to purified mesenchymal stem cell antigens;

b) adding said monoclonal antibodies to the sample;

c) measuring the antigen-monoclonal antibody reaction in the sample.

32. The method of claim 31, wherein the monoclonal antibodies that bind specifically to the mesenchymal stem cells are labelled.

33. The method of claim 31, wherein said monoclonal antibodies are produced by a hybridoma selected from the group consisting of HB 10743, HB 10744, and HB 10745.

34. A method of detecting a mesenchymal stem cell in a sample comprising contacting said sample with monoclonal antibodies produced by a hybridoma selected from the group consisting of HB 10743, HB 10744, and HB 10745 which specifically bind to mesenchymal stem cells and detecting the formation of complexes between said monoclonal antibodies and said mesenchymal stem cells.

35. A method of detecting mesenchymal stem cells in a sample comprising the steps of:

a) preparing monoclonal antibodies that bind specifically to purified mesenchymal stem cell antigens;

b) preparing second antibodies to said monoclonal antibodies;

c) labelling said second antibodies;

d) adding the monoclonal antibodies to the sample;

e) incubating the sample containing the monoclonal antibodies;

f) adding the second labelled antibodies to the sample containing the monoclonal antibodies; and,

g) measuring the antigen-monoclonal antibody-second labelled antibody reaction in said sample.

36. A composition comprising a hybrid continuous cell line that produces antibody to mesenchymal stem cells which comprises a cell hybrid of CB6F/J mouse spleen cell immunized with purified mesenchymal stem cells fused to a mouse myeloma cell and culture medium for said hybrid.

37. The composition of claim 36, wherein said antibody is an IgG fraction.

38. The composition of claim 36, wherein said culture medium is HAT medium.

39. The composition of claim 36, wherein said mouse myeloma cell is a SP 2/0 myeloma cell.

40. A method for producing a population of mesenchymal stem cells comprising the steps of:

a) providing a cell suspension of tissue containing bone marrow;

b) contacting the cell suspension with monoclonal antibodies which recognize an epitope on mesenchymal stem cells but do not recognize an epitope on hemopoietic cells; and,

c) separating and recovering from the cell suspension the cells bonded by the monoclonal antibodies.

41. The population of mesenchymal stem cells produced through the method of claim 40.

42. A method for producing a population of mesenchymal stem cells comprising the steps of:

a) providing a cell suspension of tissue containing bone marrow;

b) contacting the cell suspension with solid-phase linked monoclonal antibodies which recognize an epitope on the cell surface of mesenchymal stem cells but do not recognize an epitope on hemopoietic cells;

c) separating the unbound cells from the solid-phase linked monoclonal antibodies; and,

d) recovering the bound cells from the solid linked monoclonal antibodies.

43. The population of mesenchymal stem cells produced through the process of claim 42.

44. A suspension of human cells comprising mesenchymal stem cells substantially free of hemopoietic cells.

FIGURE 1

FIGURE 2A

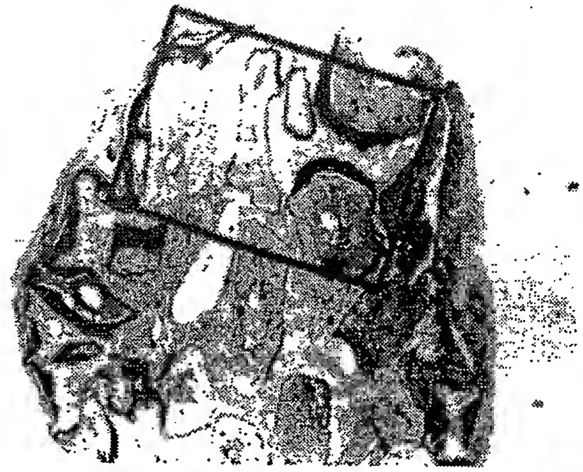


FIGURE 2B

FIGURE 2C

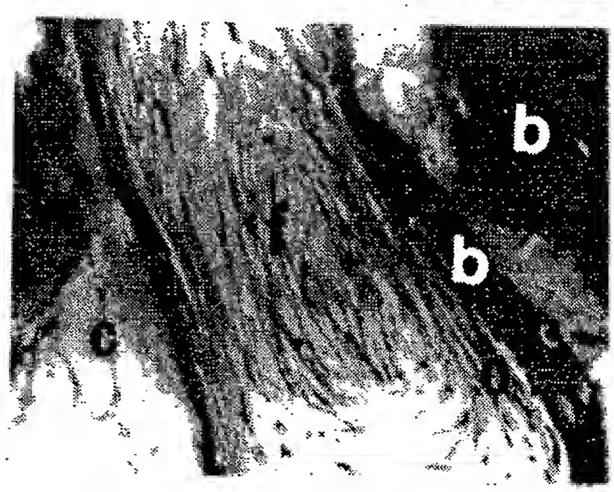


FIGURE 3B

FIGURE 3A

FIGURE 4B

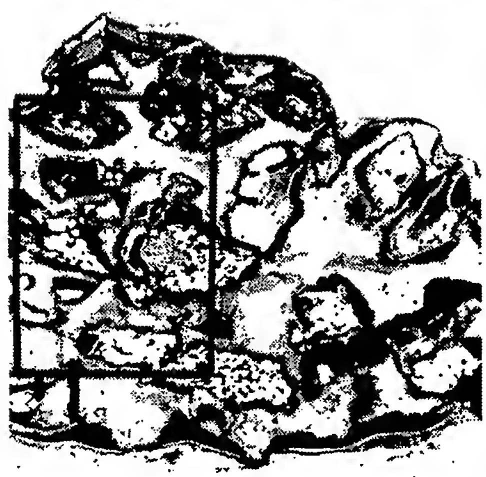


FIGURE 4A

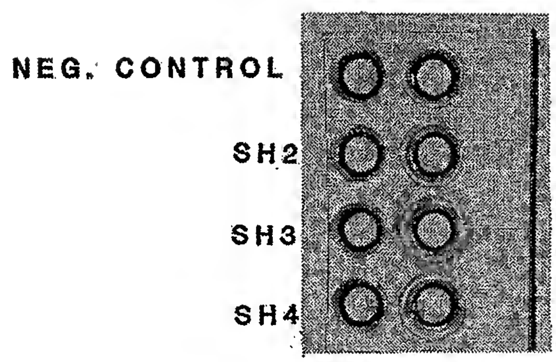
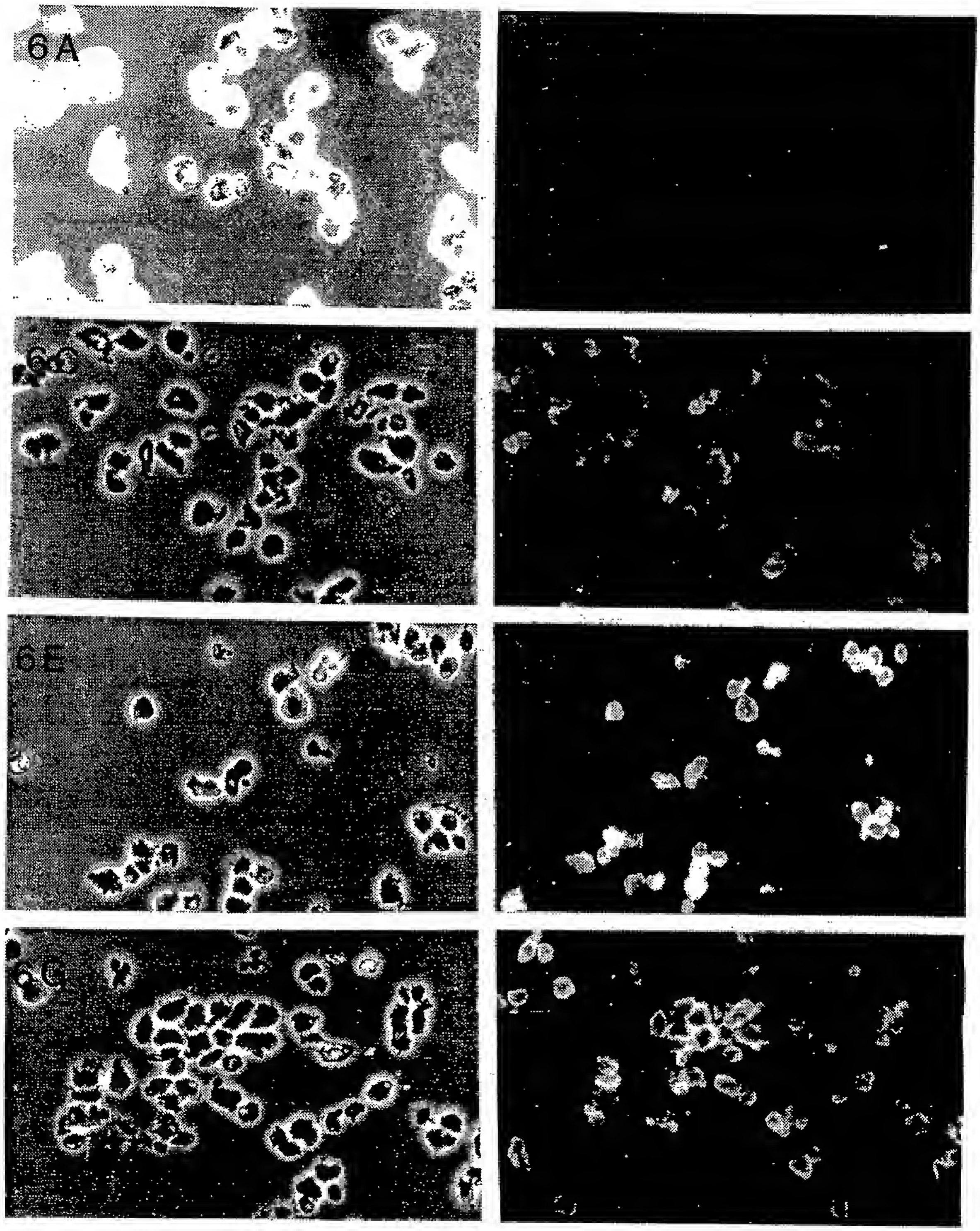
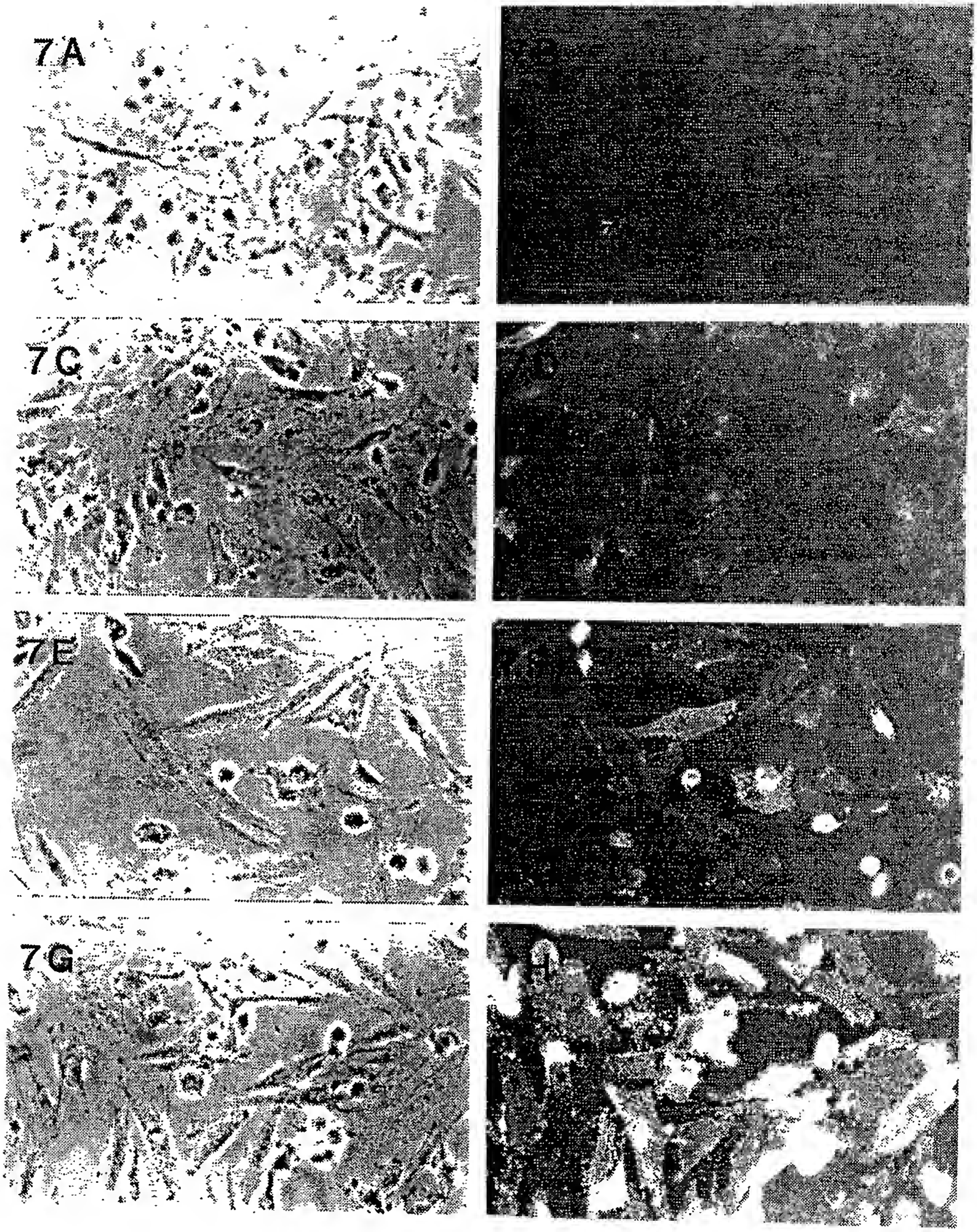


FIGURE 5


6A
6E


7A
7C
7E
7G

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/05187

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/28; C12N 5/20; C12P 21/08; C12Q 1/24; G01N 33/53, 3/554
US CL :530/388.2; 435/240.27, 70.21, 7.21, 7.95, 30, 820; 436/63, 519
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.2; 435/240.27, 70.21, 7.21, 7.95, 30, 820; 436/63, 519

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, MEDLINE, DIALOG, APS, WPI
Search terms: Antibodies, Bone Marrow, Mesenchyme, Mesoderm

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Differentiation, Volume 36, issued 1987, A. Franzen et. al., "Evidence for sequential appearance of cartilage matrix proteins in developing mouse limbs and in cultures of mouse mesenchymal cells", 199-210, especially pages 201-203.	1-15, 18, 26, 36-39
Y	Biochemical Biophysical Research Communications, Volume 92, No.2, issued 1980, T.F. Linsenmayer et al., "Monoclonal antibodies to connective tissue macromolecules: type II collagen", pages 440-446, see entire article.	1-15,18,26, & 36-39
Y	Calcified Tissue International, Volume 36, issued 1984, B. Ashton et al., "Distribution of fibroblastic colony-forming cells in rabbit bone marrow and assay of their osteogenic potential by an in vivo diffusion chamber method", pages 83-86, see entire article.	16,17,19,25-27,35,& 40-44
Y	Bone and Mineral, Volume 11, issued 1990, S. Bruder et al., "A monoclonal antibody against the surface of osteoblasts recognizes alkaline phosphatase in bone, liver, kidney, and intestine", pages 141-151, see entire article.	1-15,18,26, & 36-39

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 31 August 1992	Date of mailing of the international search report 16 SEP 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JACQUELINE G. KRIKORIAN
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	E. Harlow et al., "Antibodies: A Laboratory Manual", Volume 1, published 1988, Cold Spring Harbor Laboratory, pages 153-154, 392-393, and 578-581.	16,17,19,25-27,35, & 40-44
Y	J.W. Goding, "Monoclonal Antibodies: Principles and Practice", Volume 1, published 1983, Academic Press Inc., pages 56 to 97.	16,17,19,25-27,35, &40-44
Y	D.M. Weir et al., "Immunochemistry", Volume 1, published 1986, Blackwell Scientific Publications, pages 22.1 to 22.24.	40-44